DESCRIPTION

Title of the Invention: METHOD FOR PRODUCING SHEET-LIKE PANCREATIC ISLET

Technical Field

[0001]

The present invention relates to a production method of a sheet-like pancreatic islet having resistance to low oxygen conditions, a pancreatic islet culture, and a kit for producing a sheet-like pancreatic islet.

Background Art

[0002]

Cell-based therapy utilizing pancreatic islet has been developed as a promising novel approach for treating insulin-dependent diabetes (DM). As compared to the total organ transplantation of pancreas, an islet cell-based treatment is advantageous in that it makes minimum insult and stay in the hospital after treatment can be short. In recent international clinical trials, it has been reported that 44% of DM patients one year after the transplantation of islet cells successfully recovered insulin production, and stably maintained glycemic profile. However, two years after the transplantation, the survival rate of the transplanted islet cells drastically decreased to 14%. It is evident, therefore, that optimization of the conditions for maximizing the life extension of the transplanted cell lineage is necessary for advancing the pancreatic islet-based therapy of DM. Therefore, various culture methods have been studied to increase the function and survival rate of pancreatic islet to be used for transplantation.

[0003]

In conventional general in vitro culture methods of pancreatic islet, the cells in the pancreatic islet collected from the body are deprived of the oxygen supply system performed in vivo and maintained in the form of a clump, and therefore, they are not supplied with sufficient oxygen and the

pancreatic islet function disappears.

[0004]

To solve the above-mentioned problem, a technique for subcutaneously transplanting an islet cell sheet has been developed (non-patent document 1). In this technique, an isolated pancreatic islet is treated with trypsin-EDTA to disperse the islet cells to a single cell state, the obtained pancreatic islet cells are plated on a plate coated with laminin-5, and the culture temperature is lowered to 20°C for 20 min after the cell reached confluency, whereby the pancreatic islet cells can be recovered as a uniformly-spread tissue sheet.

[0005]

In the meantime, E-cadherin (E-cad) is a Ca^{2+}-dependent cell-cell adhesion molecule (non-patent documents 2, 3), and is essential for intercellular adhesion and colony formation of mouse embryonic stem cells (ES cell) (non-patent documents 4, 5).

[0006]

It has been reported that mouse and human ES cells can be successfully maintained on a dish coated with a fusion protein composed of the extracellular domain of E-cadherin and IgG Fc domain (non-patent document 6) (patent documents 1, non-patent documents 7, 8, 9). Although mouse ES cells do not form a colony on a culture dish coated with an E-cad-Fc fusion protein, they maintain pluripotency and can generate a germ line competent chimera mouse (non-patent documents 7, 8). In these general culture methods of ES cells, when single-celled ES cells are added, cell-to-cell adhesion occurs on a culture dish to form a clump. However, on a culture dish coated with an E-cad-Fc fusion protein, the single-celled ES cells can be cultured as they are. In addition, differentiation of mouse ES cells to liver cells on a culture dish coated with a mouse E-cad-Fc fusion protein has been reported (non-patent document 10).

[Document List]

[patent document]

[0007]

patent document 1: WO2005/090557

[non-patent documents]

[0008]

non-patent document 1: Biomaterials, vol.30, pp.5943-5949, 2009

non-patent document 2: Curr Opin Cell Biol, vol.7, pp.619-627, 1995

non-patent document 3: The Journal of Cell Biology, vol.148, p.399-404, 2000

non-patent document 4: Development, vol.122, pp.3185-3194, 1996

non-patent document 5: Stem Cells, vol.22, pp.275-282, 2004

non-patent document 6: Protein Engineering, vol.6, no.4, pp.243-245, 2003

non-patent document 7: PLos ONE, issue 1, e15, 2006

non-patent document 8: The Journal of Biological Chemistry, vol.283, no.39, pp.26468-26476, 2008

non-patent document 9: BMC Developmental Biology, vol.10, 60, 2010

non-patent document 10: Biomaterials, vol.32, no.8, pp.2032-2042, 2011

non-patent document 11: Cell Transplant., vol.18, no.5, pp.541-547, 2009

SUMMARY OF THE INVENTION

Problems to be Solved by the Invention

[0009]

Conventionally, when a sheet-like pancreatic islet is prepared by planarizing the pancreatic islet, an enzyme treatment of pancreatic islet with trypsin-EDTA to give single cells is essential to meet the physical requirements. However, pancreatic islet has been reported to have a problem that glucose responsiveness remarkably decreases due to the influence of trypsin (non-patent document 11), and the sugar responsiveness of the sheet-like pancreatic islet obtained by

this method is considered to decrease.

[0010]

The present invention aims to resolve the problem of decrease of glucose responsiveness, and provide a method of producing a sheet-like pancreatic islet having resistance to low oxygen conditions.

Means of Solving the Problems

[0011]

In an attempt to solve the above-mentioned problem, the present inventors first cultured pancreatic islet directly as a clump on a non-treated plate, without a trypsin treatment. However, the pancreatic islet was still in the form of a clump and did not take a sheet-like structure. In addition, this culture method was considered to easily induce cell death under low oxygen conditions, since it lacks the oxygen supply system to the cells in pancreatic islet, which is present in vivo. Therefore, a sheet-like structure having resistance to low oxygen conditions is preferable. However, when a sheet-like pancreatic islet is prepared using a general trypsin-EDTA treatment to form single cells, the glucose responsive function is considered to markedly decrease, since the cell-cell adhesion that controls the glucose responsive function is lost. Therefore, they intensively studied the culture conditions and found that the pancreatic islet shows a sheet-like structure by culturing the pancreatic islet on a culture dish wherein a polypeptide comprising an EC1 domain of E-cadherin and having a binding ability to said E-cadherin is fixed on or applied to a surface of a solid phase, even without dispersing the pancreatic islet to single cells by a trypsin treatment, that a pancreatic islet in a sheet-like tissue form shows higher glucose responsiveness than a pancreatic islet forming a clump and has resistance to low oxygen conditions. Further studies have resulted in the completion of the present invention.

[0012]

Accordingly, the present invention relates to the

following.

[1] A method of producing a sheet-like pancreatic islet, comprising culturing an isolated pancreatic islet in a culture vessel, wherein a polypeptide comprising an EC1 domain of E-cadherin and having a binding ability to said E-cadherin is fixed on or applied to a surface of a solid phase, while being adhered to the solid phase surface for a period sufficient for the pancreatic islet to take a sheet-like form.

[2] The production method of [1], wherein the polypeptide comprises an extracellular domain of E-cadherin.

[3] The production method of [1], wherein the polypeptide is a fusion polypeptide comprising an extracellular domain of E-cadherin and an Fc region of immunoglobulin.

[4] A pancreatic islet culture, comprising a culture vessel, wherein a polypeptide comprising an EC1 domain of E-cadherin and having a binding ability to said E-cadherin is fixed on or applied to a surface of a solid phase, and a sheet-like pancreatic islet that can be cultured in a state wherein the sheet-like pancreatic islet adheres to the polypeptide.

[5] A kit for producing a sheet-like pancreatic islet, comprising a culture vessel, wherein a polypeptide comprising an EC1 domain of E-cadherin and having a binding ability to said E-cadherin is fixed on or applied to a surface of a solid phase, and an isolated pancreatic islet.

Effect of the Invention

[0013]

Using the method of the present invention, a sheet-like pancreatic islet having resistance to low oxygen conditions can be produced without performing a trypsin treatment while suppressing a decrease in the glucose responsive function compared to conventional culture methods.

In general, the pancreatic islet does not show oxygen shortage since blood vessel induction into the pancreatic islet tissue occurs in the body. However, in the case of a transplanted pancreatic islet, the cell death of the pancreatic

islet may be induced by oxygen shortage under the environment of low oxygen conditions at the transplantation site. Using the sheet-like pancreatic islet obtained by the method of the present invention, oxygen can be efficiently supplied to each cell even in a low oxygen state, and the cell death of the pancreatic islet is suppressed even under low oxygen conditions. Moreover, since a sheet-like form of pancreatic islet is considered to increase the glucose responsive function, it is advantageous for the transplantation therapy.

Brief Description of the Drawings

[0014]

Fig. 1 shows the morphology of pancreatic islet cultured on an E-cad-Fc-coated dish and a non-treated dish at respective culture days with or without a trypsin treatment.

Fig. 2 shows the stimulation index of pancreatic islet on a non-treated dish or an E-cad-Fc-coated dish at respective culture days.

Fig. 3 shows (A) cell morphology of pancreatic islet and (B) Stimulation Index (n=3, mean±S.D., *$p<0.05$) under respective culture conditions 11 days after the culture.

Fig. 4 shows phase contrast images of pancreatic islet at respective oxygen concentrations and the observation results of cell death by PI staining, and the total cell number by DAPI staining.

Fig. 5 shows the cell death rate of the pancreatic islet cultured on an E-cad-Fc coated dish and a non-treated dish.

Description of Embodiments

[0015]

The present invention provides a method of producing a sheet-like pancreatic islet, comprising culturing an isolated pancreatic islet in a culture vessel, wherein a polypeptide comprising an EC1 domain of E-cadherin and having a binding ability to said E-cadherin is fixed on or applied to a surface of a solid phase, while being adhered to the solid phase surface for a period sufficient for the pancreatic islet to

take a sheet-like form.

[0016]

Pancreatic islet is a cell clump interspersed in the parenchyma of pancreas, which contains α cell (A cell) that secretes glucagon, β cell (B cell) that secretes insulin and δ cell (D cell) that secretes somatostatin.

[0017]

The pancreatic islet to be used in the present invention is isolated from a mammal. Examples of the mammal include, but are not limited to, laboratory animals such as rodents such as mice, rats, hamsters and guinea pigs, and rabbits; domestic animals such as pigs, bovines, goat, horses, sheep and minks; companion animals such as dogs and cats; primates such as humans, monkeys, cynomolgus monkey, rhesuses, marmosets, orangutans and chimpanzees; and the like. The mammal is preferably rodents (mouse etc.) or primates (human etc.).

[0018]

A pancreatic islet can be isolated from a mammalian pancreas by a method known per se using collagenase digestion. For example, it can be isolated by static collagenase digestion and subsequent centrifugation in Ficoll-Conray gradient (Sutton, R., 1986, Transplantation, 42:689-691/Ohtsuka, K., et. al., 1997, Transplantation, 64: 633-639).

[0019]

When the pancreatic islet is dispersed to a single cell state by a treatment with a protease such as trypsin and the like, the resulting sheet-like pancreatic islet may have reduced glucose responsiveness. In the production method of the present invention, therefore, pancreatic islet in a cell clump state is cultured in a culture vessel, wherein a polypeptide comprising an EC1 domain of E-cadherin and having a binding ability to said E-cadherin is fixed on or applied to a surface of a solid phase, without being dispersed to a single cell state by protease. The cell clump means a state wherein plural cells form one clump by mutually adhering and the like.

Depending on the mammalian species, about 10 - 10000 islet cells are generally contained in one pancreatic islet. Therefore, the number of islet cells contained in a pancreatic islet (cell clump) subjected to the above-mentioned culture is also generally within the range of 10 - 10000.

[0020]

In the present invention, an isolated pancreatic islet is cultured in a culture vessel, wherein a polypeptide comprising an EC1 domain of E-cadherin and having a binding ability to said E-cadherin is fixed on or applied to a surface of a solid phase. As a result, E-cadherin expressed on a surface of the cells constituting the pancreatic islet binds to the polypeptide, which in turn results in the adhesion of the pancreatic islet to the surface of the solid phase.

[0021]

E-cadherin is a known adhesion molecule involved in a Ca^{2+}-dependent intercellular adhesion·binding called adhesion binding or adherens junction. E-cadherin is widely expressed in parenchymal cells of internal organs such as liver, kidney, lung and the like, epithelial cells such as keratinocyte and the like, and known to be an important adhesion molecule responsible for intercellular adhesion thereof (Mareel et al., Int. J. Dev. Biol. 37: 227, 1993; Mays et al., Cord Spring Harb. Symp. Quant. Biol. 60: 763, 1995; El-Bahrawy & Pignatelli, Microsc. Res. Tech. 43:224, 1998; Nollet et al., Mol. Cell. Biol. Res. Commun. 2: 77, 1999).

[0022]

E-cadherin to be used in the method of the present invention is generally derived from a mammal. Examples of the mammal include, but are not limited to, laboratory animals such as rodents such as mice, rats, hamsters and guinea pigs, and rabbits; domestic animals such as pigs, bovines, goat, horses, sheep and minks; companion animals such as dogs and cats; primates such as humans, monkeys, cynomolgus monkey, rhesuses, marmosets, orangutans and chimpanzees; and the like. The

mammal is preferably rodents (mouse etc.) or primates (human etc.).

[0023]

with respect to each polypeptide or polynucleotide to be used in the present invention, "derived from organism X" means that the amino acid sequence or nucleic acid sequence of the polypeptide or polynucleotide has the same or substantially the same amino acid sequence or nucleic acid sequence as the amino acid sequence or nucleic acid sequence of the polypeptide or polynucleotide naturally expressed in organism X. The "substantially the same" means that the amino acid sequence or nucleic acid sequence taken note of has not less than 70% (preferably not less than 80%, more preferably not less than 90%, still more preferably not less than 95%, most preferably not less than 99%) identity with the amino acid sequence or nucleic acid sequence of a factor naturally expressed in organism X, and that the function of the factor is retained.

[0024]

As E-cadherin to be used in the present invention, a pancreatic islet derived from an animal of the same species as the pancreatic islet to be the culture target is preferable. For example, when the present invention is practiced using a pancreatic islet isolated from a mouse, E-cadherin of the mouse is desirably used. In addition, when the present invention is practiced using a pancreatic islet isolated from human, E-cadherin of the human is desirably used. However, an E-cadherin derived from a heterogeneous animal can also be used as long as a sheet-like pancreatic islet can be produced by the production method of the present invention.

[0025]

Amino acid sequences and cDNA sequences of many E-cadherins derived from mammals are known. Representative cDNA sequence and amino acid of human E-cadherin are shown in SEQ ID NOs: 1 and 2, respectively, and representative cDNA sequence and amino acid of mouse E-cadherin are shown in SEQ ID NOs: 3

and 4, respectively.

[0026]

E-cadherin is known to homophilically bind via an extracellular region (i.e., with the same molecules). The extracellular region of E-cadherin contains 5 repeat structures consisting of about 110 amino acid residues, which are regions so-called Extracellular Cadherin (EC) domains. For example, in the case of human E-cadherin (SEQ ID NO: 2), each domain of EC1, EC2, EC3, EC4, EC5 corresponds to 157 - 262, 265 - 375, 378 - 486, 487 - 595, 596 - 700, respectively (number shows the number of residues in the amino acid sequence of SEQ ID NO: 2). In the case of mouse E-cadherin (SEQ ID NO: 4), each domain of EC1, EC2, EC3, EC4, EC5 corresponds to 159 - 264, 267 - 377, 380 - 488, 489 - 597, 598 - 702, respectively (number shows the number of residues in the amino acid sequence of SEQ ID NO: 4).

[0027]

In general, since the domain (EC1) positioned at the most N-terminal side of cadherin molecule defines the binding specificity of the molecule, i.e., homophilic binding (Nose et al., Cell 61: 147, 1990), the polypeptide to be used in the present invention contains at least the EC1 domain of E-cadherin, and has a binding ability to E-cadherin. In a preferable embodiment, the polypeptide to be used in the present invention contains, in addition to the EC1 domain, one, preferably 2, more preferably 3, still more preferably 4, domains selected from EC2 - 5. In a more preferable embodiment, the polypeptide to be used in the present invention contains an extracellular region of E-cadherin. In the case of human E-cadherin, the extracellular region corresponds to the 1st - 697th amino acids of the amino acid sequence shown by SEQ ID NO: 2. In the case of mouse E-cadherin, the extracellular region corresponds to the 1st - 699th amino acids of the amino acid sequence shown by SEQ ID NO: 4.

[0028]

The polypeptide to be used in the present invention may

be a fusion polypeptide containing a sequence derived from E-cadherin and a sequence derived from other protein or peptide. For example, a polypeptide can be purified easily and efficiently by preparing the polypeptide as a fusion polypeptide with Fc region of immunoglobulin or GST (Glutathione-S-Transferase) protein, MBP (Mannose-Binding Protein) protein, avidin protein, His (oligo·histidine) tag, HA (HemAgglutinin) tag, Myc tag, VSV-G (Vesicular Stromatitis Virus Glycoprotein) tag and the like, and using protein A/G column, specific antibody column and the like. Particularly, Fc fusion polypeptide is preferable for practicing the present invention since an ability to adsorb to a culture material using polystyrene and the like is enhanced.

[0029]

Many genes encoding the Fc regions of immunoglobulin have already been isolated and identified in mammals including human. There are also many reports on the base sequences thereof and, for example, the sequence information of the base sequences of Fc regions of human IgG1, IgG2, IgG3, and IgG4 is available from public DNA databases such as NCBI and the like, and registered as accession numbers: AJ294730, AJ294731, AJ294732 and AJ294733, respectively. Therefore, those of ordinary skill in the art can obtain and use a cDNA encoding an Fc region by designing a primer or probe specific to an Fc region and using a general molecular biological method. In this case, while the animal species and subtype of a gene encoding an Fc region to be used are not particularly limited, a gene encoding an Fc region of human IgG1 or IgG2, mouse IgG2a or IgG2b and the like showing strong affinity for protein A/G is preferable. In addition, a method of enhancing the affinity for protein A by introducing a mutation into the Fc region is also known (Nagaoka et al., Protein Eng. 16: 243, 2003), and an Fc protein added with a genetic modification by this method can also be used.

[0030]

Examples of the polypeptide preferably used in the present invention include polypeptide containing an extracellular region of E-cadherin disclosed in Nagaoka et al., Biotechnol. Lett. 24: 1857, 2002 and Protein Eng. 16: 243, 2003.

[0031]

In addition, purified recombinant proteins (Recombinant Human/Mouse E-cadherin-Fc Chimera; R&D systems, Genzyme Techne) prepared by introducing a fusion gene wherein a sequence encoding an Fc region of human IgG and a cDNA of His-tag sequence are linked to a cDNA encoding a mouse or human extracellular region of E-cadherin into mouse cells and expressing same are commercially available, and these can also be applied to the present invention. Moreover, a culture dish having the bottom coated with E-cadherin-Fc is commercially available from SUMITOMO BAKELITE CO., LTD. and the like, and this can also be applied to the present invention.

[0032]

The above-mentioned polypeptide is preferably isolated or purified. Being "isolated or purified" means being artificially placed in a state different from that naturally present, for example, an operation to remove components other than the object component from the naturally-present state has been applied. The purity of the isolated or purified the above-mentioned polypeptide (proportion of the above-mentioned polypeptide weight to the total polypeptide weight) is generally not less than 30%, preferably not less than 50%, more preferably not less than 70%, still more preferably not less than 90% (e.g., 100%).

[0033]

The above-mentioned polypeptide can be produced by culturing mammalian cells such as COS cell, 293 cell, CHO cell and the like introduced with an expression vector capable of expressing the polypeptide, and isolating and purifying the polypeptide from the culture by a biochemical method known per se. In the expression vector, a nucleic acid (DNA etc.)

encoding the polypeptide is linked to a nucleic acid sequence enabling the transcription and expression of genes in a wide range of mammalian cells, what is called a promoter sequence, in a manner enabling transcription and expression under the regulation of the promoter. The gene to be transcribed and expressed is desirably linked with polyA addition signal. Preferable promoter includes promoters derived from virus such as SV (Simian Virus) 40 virus, cytomegalovirus (CMV), Rous sarcoma virus and the like, β-actin promoter, EF (Elongation Factor) 1α promoter and the like.

[0034]

The material for constituting a solid phase in a culture vessel is not particularly limited as long as it can achieve production of a sheet-like pancreatic islet when used for the production method of the present invention, a material having no cytotoxicity, permitting sterilization and having affinity for protein can be generally used. In general, plastic or glass materials are preferable. The material may be a metal or ceramic, and is not limited to a certain material.

[0035]

The plastic material is a thermosetting or thermoplastic polymer superior in moldability and, for example, polystyrene, methacrylic resin, polymethylpentene, ethylene-vinylalcohol copolymer, polypropylene, cellulose, polyethylene, polysulfone, polyacrylonitrile and the like can be used without limitation thereto.

[0036]

The glass material means one resulting from vitrification of silicate, borate, phosphate and the like without crystallization. Since vitrification tendency is strong, silicate glass is preferable. In addition, crystallized glass which is one kind of a composite material produced by heat-treating silicate glass is more preferable since it has rich moldability and high impact resistance.

[0037]

Examples of the culture vessel include, but are not limited to, petri dish, plate, flask, bottle and the like. The form of the culture vessel is not particularly limited as long as the pancreatic islet adheres to a solid phase surface and can achieve production of a sheet-like pancreatic islet when applied to the method of the present invention.

[0038]

The solid phase surface refers to a part enabling adhesion of a pancreatic islet to be cultured to a solid phase, when the pancreatic islet is cultured while being adhered to the solid phase in a culture vessel, for example, a part to be in contact with a medium when the medium is added.

[0039]

As a method for fixing or coating a polypeptide onto a solid phase surface, a method using a non-covalent bond (hydrogen bond, ionic bond, hydrophobic bond etc.), a covalent bond and the like can be generally used.

[0040]

Examples of the method for fixing or coating a polypeptide onto a solid phase surface by using a non-covalent bond include a method of standing still the solid phase surface in a suitable buffer (e.g., phosphate buffer etc.) containing the polypeptide. The conditions (buffer type, concentration of polypeptide in buffer, standing time etc.) of the method can be appropriately determined as long as it can achieve production of a sheet-like pancreatic islet when used for the production method of the present invention. For example, when a polypeptide is a fusion polypeptide containing an extracellular region of mouse or human E-cadherin and an Fc region of mouse or human IgG, and the material constituting the solid phase is a plastic (e.g., polystyrene), the polypeptide is fixed or applied onto the surface of the solid phase by standing the solid phase for about 0.5 - 24 hr in a neutral phosphate-buffered saline containing a polypeptide at a concentration of generally 0.01 - 1000 μg/mL (preferably 0.1 - 200 μg/mL, more

preferably 1 - 50 μg/mL).

[0041]

Examples of the method for fixing or coating a polypeptide by using a covalent bond include a method of introducing a functional group into a solid phase surface by treating the solid phase surface with a silane coupling agent having a functional group, and binding the polypeptide to the functional group with a crosslinking agent (see, for example, JP-A-2003-189843). Examples of the functional group that can be introduced include amino group, aldehyde group, epoxy group, carboxyl group, hydroxyl group, thiol group and the like. Examples of the silane coupling agent include γ-aminopropyltriethoxysilane, N-β-(aminoethyl)γ-aminopropyltrimethoxysilane, N-β-(aminoethyl)γ-aminopropylmethyldimethoxysilane and the like. Examples of the crosslinking agent include diethylene glycol diglycidyl ether, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, N,N'-carbodiimidazole, glutaraldehyde, anhydrous succinic acid, anhydrous phthalic acid, hexamethylenediisocyanate and the like.

[0042]

In this way, an isolated pancreatic islet is cultured in a culture vessel, wherein a polypeptide comprising an EC1 domain of E-cadherin and having a binding ability to said E-cadherin is fixed on or applied to a surface of a solid phase, whereby E-cadherin expressed on the surface of the cells constituting the pancreatic islet binds to the polypeptide, as a result of which the pancreatic islet adheres to the solid phase surface.

[0043]

While the strength of the adhesion of the pancreatic islet to the solid phase surface is not particularly limited as long as it can achieve the production of the sheet-like pancreatic islet by the production method of the present invention, it is generally a strength that prevents dissociation unless a physical and/or a chemical treatment

are/is applied. Examples of the physical treatment include a treatment by pipetting or tapping and the like. Examples of the chemical treatment include a treatment with a chelating agent such as EDTA, EGTA and the like, a treatment with a protease such as trypsin and the like, and the like.

[0044]

In the production method of the present invention, as a basal medium of a medium used for culturing a pancreatic islet, one known per se and usable for in vitro culture of pancreatic islet can be used, and is not particularly limited as long as it can achieve the production of the sheet-like pancreatic islet by the production method of the present invention. For example, DMEM, EMEM, RPMI-1640, α-MEM, F-12, F-10, M-199, HAM and the like can be mentioned. In addition, a medium altered for culturing pancreatic islet and the like may be used, and a mixture of the above-mentioned basal media may also be used.

[0045]

A medium used for culturing pancreatic islet in the production method of the present invention can contain an additive known per se and generally used for the tissue culture of pancreatic islet. While the additive is not particularly limited as long as it can achieve the production of the sheet-like pancreatic islet by the production method of the present invention, for example, growth factors (e.g., insulin etc.), iron sources (e.g., transferrin etc.), polyamines (e.g., putrescine etc.), minerals (e.g., sodium selenate etc.), saccharides (e.g., glucose etc.), organic acids (e.g., pyruvic acid, lactic acid etc.), serum proteins (e.g., albumin etc.), amino acids (e.g., L-glutamine etc.), reducing agents (e.g., 2-mercaptoethanol etc.), vitamins (e.g., ascorbic acid, d-biotin etc.), antibiotics (e.g., streptomycin, penicillin, gentamicin etc.), buffering agents (e.g., HEPES etc.) and the like can be mentioned. The additive is preferably contained in a medium at a concentration within the range known per se.

[0046]

The medium used for culturing a pancreatic islet in the production method of the present invention may contain serum. While the concentration of the serum is not particularly limited as long as it can achieve the production of the sheet-like pancreatic islet by the production method of the present invention, it is generally within the range of 0.1 - 30(v/v)%.

[0047]

As other culture conditions for a pancreatic islet in the production method of the present invention, culture conditions in common use in pancreatic islet tissue culture technology can be used. For example, culturing temperature is normally in the range of about 30-40°C, and preferably exemplified by about 37°C. CO_2 concentration is normally in the range of about 1-10%, and preferably exemplified by about 5%. Humidity is normally in the range of about 70-100%, and preferably exemplified by about 95-100%.

[0048]

In the production method of the present invention, an isolated pancreatic islet is cultured for a period sufficient for taking a sheet-like form in a culture vessel wherein the above-mentioned polypeptide is fixed on or applied to a surface of the solid phase while being adhered to the solid phase surface. When an isolated pancreatic islet is cultured in a culture vessel wherein the above-mentioned polypeptide is fixed on or applied to a surface of the solid phase, the pancreatic islet adheres to the solid phase surface, and spreads over the solid phase surface in time to take a sheet-like form. The "sheet-like" refers to a shape having a sufficiently large length or width (preferably, both) relative to the thickness of the pancreatic islet. For example, the length or width (preferably, both) of the sheet-like pancreatic islet is generally not less than 3-fold, preferably not less than 10-fold, of the thickness. The "thickness" of the pancreatic islet means the thickness of the thickest portion in the direction perpendicular to the solid phase surface of the

culture vessel. The "length" of the pancreatic islet means the maximum length in the direction orthogonal to the thickness direction of the aforementioned pancreatic islet. The "width" of the pancreatic islet means the maximum length among the lengths in the direction orthogonal to both the thickness direction and the length direction of the aforementioned pancreatic islet. In one embodiment, the sheet-like pancreatic islet includes a single layer of pancreatic islet cells. The time necessary for taking a sheet-like form varies depending on the animal species from which pancreatic islet is derived, the above-mentioned polypeptide constitution, and culture conditions, and generalization thereof is difficult. When the pancreatic islet of a mouse is cultured in a culture vessel wherein a polypeptide comprising an extracellular region of mouse E-cadherin and an Fc region of IgG is fixed on or applied to a surface of a solid phase, the pancreatic islet begins to spread in about 3 days after the start of the culture, and it takes a sheet-like form in about 6 days to 10 days. Even when a pancreatic islet of other animal species or a polypeptide with other constitution is used, those of ordinary skill in the art can appropriately determine, by reference to this culture period, a period sufficient for pancreatic islet to take a sheet-like form.

[0049]

While the upper limit of the culture period is not particularly limited as long as the obtained sheet-like pancreatic islet maintains glucose responsiveness, when the culture period becomes long, insulin secretability may decrease. Therefore, the culture period is generally within 8 weeks, preferably within 4 weeks, more preferably within 1 week.

[0050]

A sheet-like pancreatic islet obtained by the production method of the present invention shows good glucose responsiveness. The glucose responsiveness means an ability to sense an increase in the glucose concentration and secrete

insulin. For example, the insulin concentration of a culture medium after culture of a sheet-like pancreatic islet obtained by the production method of the present invention in DMEM containing 4500 mg/l of glucose at 37°C, 5% CO_2 for 1 hr is generally not less than 1.5-fold, preferably not less than 2-fold, more preferably not less than 3-fold, of that in a culture medium after culture in DMEM containing 1000 mg/l of glucose at 37°C, 5% CO_2 for 1 hr.

[0051]

In addition, the present invention provides a pancreatic islet culture containing a culture vessel, wherein a polypeptide comprising an EC1 domain of E-cadherin and having a binding ability to said E-cadherin is fixed on or applied to a surface of a solid phase, and a sheet-like pancreatic islet, which enables culture of the sheet-like pancreatic islet while it is adhered to the solid phase surface.

[0052]

In one embodiment, in the culture, the sheet-like pancreatic islet survives and functions while being adhered to the solid phase surface.

[0053]

In one embodiment, in the culture, the sheet-like pancreatic islet survives while being adhered to the solid phase surface. In another embodiment, in the culture, the sheet-like pancreatic islet grows while being adhered to the solid phase surface.

[0054]

The culture refers to a resulting product obtained by culturing tissues and cells.

[0055]

The definition and embodiment of each term relating to the culture of the present invention are the same as those described for the above-mentioned production method of the present invention.

[0056]

The culture of the present invention can contain a medium used for the aforementioned method of the present invention, insulin secreted by the pancreatic islet and the like in addition to the above-mentioned culture vessel, and the sheet-like pancreatic islet.

[0057]

The pancreatic islet culture of the present invention is useful for the practice of the regenerative medicine utilizing the sheet-like pancreatic islet.

[0058]

A kit for the production of a sheet-like pancreatic islet containing a culture vessel, wherein a polypeptide comprising an EC1 domain of E-cadherin and having a binding ability to said E-cadherin is fixed on or applied to a surface of a solid phase, and an isolated pancreatic islet is provided. Using the kit of the present invention, a sheet-like pancreatic islet can be produced easily by the above-mentioned production method of the present invention.

[0059]

The definition and embodiment of each term relating to the kit of the present invention are the same as those described for the above-mentioned production method of the present invention.

[0060]

The kit of the present invention may further contain a reagent used for the above-mentioned production method of the present invention. Examples of the reagent include proteases such as collagenase and the like used for the isolation of a pancreatic islet, medium, serum and the like.

[0061]

The contents disclosed in any publication cited in the present specification, including patents and patent applications, are hereby incorporated in their entireties by reference, to the extent that they have been disclosed herein.

[0062]

The present invention is explained in more detail in the following by referring to Examples shown below, which are not to be construed as limitative.

Examples

[0063]

[Example 1]

Pancreatic islet culture

Pancreatic islet was separated from male mice (body weight 20 - 25 g, 9 - 10-week-old, C57BL/6J; CHARLES RIVER LABORATORIES JAPAN, INC.) by a digestion method using collagenase from clostridium histdyticum Type V (GIBCO). The pancreatic islet was separated by a density gradient method using Biocoll Separating Solution (Biocheom AG) and picked up by hand using Pipetman. The separated pancreatic islet was cultured (37°C/CO_2; 5%) on a 3.5 cm E-cad-Fc-coated dish (SUMITOMO BAKELITE CO., LTD.) and a non-treated dish (IWAKI) in Dulbecco's Modified Eagle's Medium (DMEM; SIGMA) added with 10(v/v)% FBS (GIBCO), and 1(v/v)% Anti-Anti (GIBCO) as an antibiotic. The medium was changed 7 days after the pancreatic islet was plated in the culture vessel, and changed every 3 days thereafter.

[0064]

[Example 2]

Observation of pancreatic islet morphology

The separated pancreatic islet was plated on a 3.5 cm E-cad-Fc coated dish (SUMITOMO BAKELITE) and a non-treated dish (IWAKI). Furthermore, pancreatic islet to be single-celled was treated with trypsin-EDTA (GIBCO) for 5 min at 37°C, and pancreatic islet cells dispersed in a single-cell state were plated similarly. The pancreatic islet and pancreatic islet cells single-celled by a trypsin-EDTA treatment were cultured (37°C/CO_2; 5%) using Dulbecco's Modified Eagle's Medium (DMEM; SIGMA) added with 10% FBS (GIBCO), and 1(v/v)% Anti-Anti (GIBCO) as an antibiotic. The medium was changed 7 days after the pancreatic islet was plated in the culture vessel, and

changed every 3 days thereafter.

[0065]

Thereafter, the morphology of the pancreatic islet was observed by an inverted phase contrast microscope (Olympus IX-70) every other day. The results are shown in Fig. 1. When the pancreatic islet was plated on an E-cad-Fc-coated dish, the pancreatic islet spread on the dish in 3 days from the start of the culture, and a sheet-like form was observed in 6 days from the start of the culture. On the other hand, when plated on a non-treated dish (IWAKI), the tissue morphology did not change and the pancreatic islet was still in the form of a clump. Even after culture for 18 days, a sheet-like form could not be afforded. When pancreatic islet cells in a single cell state were plated on an E-cad-Fc-coated dish, adhesion of the cell to the dish was observed. On the other hand, when pancreatic islet cells in a single cell state were plated on a non-treated dish (IWAKI), the cells underwent necrosis.

[0066]

[Example 3]

Evaluation of glucose responsive function by Stimulation Index

Under the same culture conditions as in Example 1, a pancreatic islet (40 pieces) separated from a mouse was plated and cultured on a 3.5 cm E-cad-Fc-coated dish or a non-treated dish. After the start of the culture, whether the insulin secretion amount of the pancreatic islet can be controlled by the concentration of glucose in the culture medium was examined. The pancreatic islet cultured on the E-cad-Fc-coated dish or non-treated dish was washed twice with a low glucose medium (1,000 mg/l DMEM), thereafter cultured in a low glucose medium (1,000 mg/l DMEM) for 1 hr, and the amount of insulin secreted in the supernatant was taken as an insulin secretion amount with low concentration glucose. Thereafter, the pancreatic islet was washed twice with a high glucose medium (4,500 mg/l DMEM), cultured in a high glucose medium (4,500 mg/l DMEM) for 1 hr, and the amount of insulin secreted in the supernatant was

taken as an insulin secretion amount with high concentration glucose. The recovered supernatant was subjected to the measurement of an insulin secretion amount of the pancreatic islet on the non-treated dish and E-cad-Fc-coated dish, by using Levis insulin-mouse (Shibayagi Co. Ltd.), which is an ELISA (Enzyme-Linked ImmunoSorbent Assay) kit. To evaluate the function of the pancreatic islet, Stimulation Index (SI; ratio of insulin secretion amount under high concentration glucose environment to insulin secretion amount under low concentration glucose environment) was calculated (Fig. 2).

[0067]

On each culture day, the pancreatic islet showed glucose responsiveness on the non-treated dish and the E-cad-Fc-coated dish, whereby an insulin secretion ability was confirmed. As a result of the calculation of the Stimulation index, the pancreatic islet cultured on the E-cad-Fc-coated culture vessel showed a pancreatic islet function equal to or not less than that of the pancreatic islet cultured on the non-treated dish (Fig. 2).

[0068]

[Example 4]

Influence of trypsin treatment on glucose responsive function

A mouse-derived pancreatic islet (40 pieces) was plated on a 3.5 cm E-cad-Fc-coated dish or a non-treated dish. Furthermore, a mouse-derived pancreatic islet was incubated in trypsin-EDTA (GIBCO) at 37°C, 5 min to prepare pancreatic islet cells in a single-cell state, which were plated in the same manner as with pancreatic islet. At 11 days from the start of the culture, the glucose responsive function of the pancreatic islet and pancreatic islet cells was examined by SI. The evaluation by ELISA was performed by the same method as in Example 3. In addition, SI on each culture day was calculated.

[0069]

The insulin secretion amount of the pancreatic islet cells dispersed by a trypsin-EDTA treatment decreased on both

the non-treated dish and E-cad-Fc coated dish, as compared to that of a pancreatic islet without the treatment. In addition, the stimulation index of the pancreatic islet cells dispersed by a trypsin-EDTA treatment on an E-cad-Fc-coated dish also decreased as compared to that of a pancreatic islet without the treatment (Fig. 3). Therefore, it was suggested that a trypsin-EDTA treatment decreases the glucose responsive function.

[0070]

[Example 5]

Measurement of cell death induction rate in pancreatic islet under low oxygen conditions

Under the same culture conditions as in Example 1, a pancreatic islet separated from a mouse was plated and cultured for 1 week on a 3.5 cm E-cad-Fc-coated dish or a non-treated dish. The pancreatic islet on the E-cad-Fc-coated dish was collected with PBS (GIBCO) containing 1 mM EDTA (ethylenediaminetetraacetic acid), and incubated (37°C) for 1 hr under low oxygen conditions (O_2; 5%) and high oxygen conditions (O_2; 20%) in a suspension state in the same manner as with the pancreatic islet on the non-treated dish. Thereafter, the pancreatic islet was stained by incubating (37°C) for 30 min in DMEM containing DAPI Nucleic Acid Stain (DAPI; Lonza) 5 μg/ml and Propidium iodide (PI; Roche) 0.5 μg/ml and observed under a microscope (Fig. 4).

[0071]

To quantify the cell number of the pancreatic islet, the DAPI stained cell number was calculated as an index by ImageJ. Moreover, to quantify the number of the dead cells, a PI-stained cell number was calculated as an index by ImageJ. The cell death rate was calculated by the following formula:

Cell death rate(%)=(cell number stained with PI/cell number stained with DAPI)×100

[0072]

The results are shown in Figs. 4 and 5. When the oxygen

concentration was 20%, the cell death induction rate was almost of the same level on the non-treated dish and E-cad-Fc-coated dish. However, when the oxygen concentration was 5%, the pancreatic islet on the non-treated dish showed positive in PI staining from the central part and the cells underwent necrosis (Fig. 4), which confirms a high cell death induction rate as compared to the pancreatic islet on the E-cad-Fc coated dish (Fig. 5). From the above, it was suggested that the cell death of the pancreatic islet cells induced under low oxygen conditions is suppressed by culturing on an E-cad-Fc-coated dish.

Industrial Applicability

[0073]

Using the method of the present invention, a sheet-like pancreatic islet having resistance to low oxygen conditions can be produced without performing a trypsin treatment while suppressing a decrease in the glucose responsive function.

In general, the pancreatic islet does not show oxygen shortage since blood vessel induction into the pancreatic islet tissue occurs in the body. However, in the case of a transplanted pancreatic islet, the cell death of the pancreatic islet may be induced by oxygen shortage under the environment of low oxygen conditions at the transplantation site. Using the sheet-like pancreatic islet obtained by the method of the present invention, oxygen can be efficiently supplied to each cell even in a low oxygen state, and the cell death of the pancreatic islet is suppressed even under low oxygen conditions, and therefore, it is advantageous for the transplantation therapy.

[0074]

This application is based on a patent application No. 2011-086336 filed in Japan (filing date: April 8, 2011), the contents of which are incorporated in full herein.

Claims

1. A method of producing a sheet-like pancreatic islet, comprising culturing an isolated pancreatic islet in a culture vessel, wherein a polypeptide comprising an EC1 domain of E-cadherin and having a binding ability to said E-cadherin is fixed on or applied to a surface of a solid phase, while being adhered to the solid phase surface for a period sufficient for the pancreatic islet to take a sheet-like form.

2. The production method according to claim 1, wherein the polypeptide comprises an extracellular domain of E-cadherin.

3. The production method according to claim 1, wherein the polypeptide is a fusion polypeptide comprising an extracellular domain of E-cadherin and an Fc region of immunoglobulin.

4. A pancreatic islet culture, comprising a culture vessel, wherein a polypeptide comprising an EC1 domain of E-cadherin and having a binding ability to said E-cadherin is fixed on or applied to a surface of a solid phase, and a sheet-like pancreatic islet that can be cultured in a state wherein the sheet-like pancreatic islet adheres to the polypeptide.

5. A kit for producing a sheet-like pancreatic islet, comprising a culture vessel, wherein a polypeptide comprising an EC1 domain of E-cadherin and having a binding ability to said E-cadherin is fixed on or applied to a surface of a solid phase, and an isolated pancreatic islet.