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HUMAN FATTY-LIVER MODEL CELLS FOR USE IN SCREENING METHOD

Abstract

An object of the present invention is to provide human fatty-liver model cells exhibiting symptoms of fatty-liver tissue that can be used for a method for screening for a substance having an effect on dyslipidemia including fatty liver and the present invention provides human fatty-liver model cells for use in a method for screening for a substance having an effect on dyslipidemia based on an increase/decrease of very low density lipoprotein (VLDL) in culture supernatant as an index, in which the culture supernatant of the cells contains VLDL and low density lipoprotein (LDL), in which the VLDL is contained more than the LDL.

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Background/Summary

CROSS-REFERENCE TO RELATED APPLICATIONS [0001] The present application claims priority under 35 U.S.C. § 371 to International Patent Application No. PCT/JP2022/007494, filed Feb. 24, 2022, which claims priority to and the benefit of Japanese Patent Application No. 2021-030911, filed Feb. 26, 2021. [0002] The contents of these applications are hereby incorporated by reference in their entireties.

TECHNICAL FIELD

[0003] The present invention relates to human fatty-liver model cells for use in a method for screening for a substance having an effect on dyslipidemia such as fatty liver based on an increase/decrease of very low density lipoprotein (VLDL) in the culture supernatant as an index.

BACKGROUND ART

[0004] Fatty liver is a collective term referring to diseases producing liver disorder, which is caused by excessive accumulation of lipid such as neutral fat within hepatocytes. In fatty liver, accumulation of fat droplets is observed in a $\frac{1}{3}$ or more area of the hepatocytes constituting the liver lobule. The occurrence of frequency of fatty liver likely increases year by year due to change in eating and lifestyle habits. In past days, it was considered that fatty liver may leave alone; however, in recent years, nonalcoholic fatty liver disease (NAFLD) has attracted attention; and cases where fatty liver develops into non-alcoholic steatohepatitis (NASH) and further into cirrhosis/liver cancer, have been found. Because of this, it has been required to suitably treat fatty liver. Attempts to functionally analyze its pathology and develop effective therapeutic agents have been made.

[0005] For investigating the onset mechanism of fatty liver and prevention/treatment thereof, non-human animal models exhibiting fatty-liver symptoms have been prepared. For example, Patent Literature 1 discloses that a non-human animal model exhibiting fatty-liver symptoms is prepared by transplanting human hepatocytes to an immunodeficient non-human animal with a liver disorder. In the non-human animal model, symptoms of fatty liver, such as large fat droplets and hepatic steatosis, are observed.

CITATION LIST

Patent Literature

[0006] Patent Literature 1: WO2008/001614

SUMMARY OF INVENTION

Technical Problem

[0007] Non-human animal models have the following problems. A great deal of time and labor/cost are required for preparing, raising and managing the animal models. In addition, individual difference and reproducibility, and ethical limitations in use are also problems. For the reason, in order to efficiently investigate the onset mechanism of fatty liver and prevention/treatment for fatty liver, development of an in vitro evaluation system for human fatty liver, more specifically, human fatty-liver model cells, is strongly desired.

[0008] When the present inventors cultured human hepatocytes derived from fatty liver in vitro, they found that fat droplets disappear, and thus, the human hepatocytes cannot maintain the symptoms of fatty liver. More specifically, they excised out fatty liver from a non-human animal model showing the aforementioned symptoms of fatty liver, separated/collected human hepatocytes showing symptoms of fatty liver such as accumulation of fat droplets from the fatty liver, and

cultured the human hepatocytes in vitro. As a result, they found that fat droplets disappear from the human hepatocytes, and thus, the human hepatocytes cannot maintain the symptoms of fatty liver. [0009] Accordingly, an object of the present invention is to provide novel human fatty-liver model cells that enables human hepatocytes derived from fatty liver to maintain the symptoms of fatty liver such as accumulation of fat droplets and that can be used in a method for screening for a substance having an effect on dyslipidemia such as fatty liver.

Solution to Problem

[0010] The present inventors conducted intensive studies with a view to attaining the aforementioned objects. As a result, accumulation of fat droplets, lipid secretion/accumulation, expression of fatty liver related genes and others were observed by culturing human hepatocytes derived from fatty liver in a medium containing dimethyl sulfoxide. They found that human fatty-liver model cells maintaining the symptoms of fatty liver can be obtained. They also have found that the culture supernatant of human fatty-liver model cells contains very low density lipoprotein (VLDL) and low density lipoprotein (LDL), in which the VLDL is contained more than the LDL; and that a substance having an effect on dyslipidemia such as fatty liver can be screened for based on an increase/decrease of VLDL content in the culture supernatant as an index.

[0011] The present invention was attained based on these findings and has the following features.

[0012] [1] Human fatty-liver model cells for use in a method for screening for a substance having an effect on dyslipidemia such as fatty liver based on an increase/decrease of very low density lipoprotein (VLDL) in culture supernatant as an index, in which the culture supernatant of the cells contains VLDL and low density lipoprotein (LDL), in which the VLDL is contained more than the LDL. [0013] [2] The cells according to [1], which is a monolayer cell culture. [0014] [3] The cells according to [1] or [2], in which the VLDL includes Large-VLDL, Medium-VLDL and Small-VLDL, and the Large-VLDL is contained in a ratio of 70 mass % or more of total VLDLs. [0015] [4] The cells according to any one of [1] to [3], which are obtained by culturing human hepatocytes derived from fatty liver in a medium containing dimethyl sulfoxide. [0016] [4-1] The cells according to any one of [1] to [4], which contain lipid 2 times or more, 3 times or more, 4 times or more, 5 times or more, 6 times or more, 7 times or more, 8 times or more, or 9 times or more as large as lipid in human hepatocytes derived from fatty liver, which are cultured in the same conditions except that dimethyl sulfoxide is not contained. [0017] [4-2] The cells according to any one of [1] to [4], which contain a lipoprotein (more preferably, triglyceride) amount contained in culture supernatant 5 times or more, 6 times or more, 7 times or more, 8 times or more, or 9 times or more as large as a lipoprotein (more preferably, triglyceride) amount contained in culture supernatant of human hepatocytes derived from fatty liver, which are cultured in the same conditions except that dimethyl sulfoxide is not contained. [0018] [5] The cells according to [4], [4-1] or [4-2], in which the human hepatocytes derived from fatty liver are collected from a chimeric non-human animal having human hepatocytes. [0019] [6] A method for screening for a substance having an effect on dyslipidemia, including the steps of: [0020] administering a test substance to the cells according to any one of [1] to [5]; and [0021] comparing very low density lipoprotein (VLDL) amount in culture supernatant between the cells to which the test substance is administered and cells to which the test substance is not administered.

[0022] The specification incorporates the contents disclosed in, for example, the specification of Japanese Patent Application 2021-30911 filed on Feb. 26, 2021 based on which the priority of the present application is claimed.

[0023] All publications, patents and patent applications cited in the specification are incorporated herein in their entirety by reference.

Advantageous Effects of Invention

[0024] According to the present invention, it is possible to provide human fatty-liver model cells, in which e.g., accumulation of fat droplets, secretion/accumulation of lipids and expression of fatty liver related genes are observed, and which is to be used in a method for screening for a substance

having an effect on dyslipidemia such as fatty liver based on an increase/decrease of very low density lipoprotein (VLDL) in the culture supernatant as an index.

Description

BRIEF DESCRIPTION OF DRAWINGS

[0025] FIG. 1 shows photographs of monolayer cell cultures of PXB-cells cultured in medium B (DMSO (+)) or medium C (DMSO (-)) for 5 days. The left photograph shows PXB-cells cultured in medium B (DMSO (+)) and the right photograph shows PXB-cells cultured in medium C (DMSO (-)).

[0026] FIG. 2 shows the measurement results of the content of total neutral fat (triglyceride) of lipoproteins contained in the culture supernatant of PXB-cells cultured in medium B (DMSO (+)) or medium C (DMSO (-)) for 5 days. The results are shown by relative values based on the content (regarded as “100”) of the total neutral fat in the culture supernatant of PXB-cells cultured in medium B (DMSO (+)).

[0027] FIG. 3 shows the measurement results of the contents of total cholesterol (left) and total neutral fat (triglyceride) (right) of lipoproteins in each of culture supernatants of PXB-cells cultured in medium B (DMSO (+)) for 5 days, 9 days, 12 days and 14 days, and HepG2 cells and HuH7 cells.

[0028] FIG. 4 shows the measurement results of the contents of total cholesterol (left) and total neutral fat (triglyceride) (right) of lipoproteins (4 types of subgroups) in each of culture supernatants of PXB-cells cultured in medium B (DMSO (+)) for 5 days, 9 days, 12 days and 14 days, and HepG2 cells and HuH7 cells.

[0029] FIG. 5 shows the measurement results of the contents of total cholesterol (left) and total neutral fat (triglyceride) (right) within PXB-cells cultured in medium B (DMSO (+)) for 5 days, 9 days, 12 days and 14 days, and HepG2 cells and HuH7 cells.

[0030] FIG. 6 shows the measurement results of expression levels of fatty liver related genes (FASN, SREBF1, G6PC) in PXB-cells cultured in medium B (DMSO (+)) for 3 days and 6 days. The results are shown by relative values based on the expression level (regarded as “1”) of each of the genes of PXB-cells cultured in medium B (DMSO (+)) for 3 days.

[0031] FIG. 7(A) shows the analysis results of neutral fat (triglyceride) content in each of subgroups of lipoprotein in the culture supernatant of PXB-cells cultured for 2 days in medium D containing 0.75 mM oleic acid, and FIG. 7(B) shows the analysis results on the neutral fat (triglyceride) contents of Large, Medium and Small VLDLs different in size, in the culture supernatant.

[0032] FIG. 8(A) shows the analysis results of neutral fat (triglyceride) content in each of subgroups of lipoprotein in the culture supernatant of PXB-cells cultured for 2 days in medium D containing 1 nM and 5 nM lomitapide, and FIG. 8(B) shows the analysis results on the neutral fat (triglyceride) contents of Large, Medium and Small VLDLs different in size, in the culture supernatant.

[0033] FIG. 9(A) shows the analysis results of neutral fat (triglyceride) content in each of subgroups of lipoprotein in the culture supernatant of PXB-cells cultured for 2 days in medium D containing 10 μ M, 50 μ M and 100 μ M fenofibrate, and FIG. 9(B) shows the analysis results on the neutral fat (triglyceride) contents of Large, Medium and Small VLDLs different in size, in the culture supernatant.

DESCRIPTION OF EMBODIMENTS

1. Human Fatty-Liver Model Cells

[0034] The human fatty-liver model cells in the present invention can be produced by a method for producing human fatty-liver model cells including a step of culturing human hepatocytes derived

from fatty liver in a medium containing dimethyl sulfoxide. Now, the method for producing human fatty-liver model cells of the present invention will be described.

[1-1] Human Hepatocytes Derived from Fatty Liver

[0035] In the present invention, “human hepatocytes derived from fatty liver” refer to human hepatocytes collected from a fatty liver tissue. The human hepatocytes can be once frozen and then thawed to put in use. The fatty liver tissue that can be used includes a fatty liver tissue derived from a human patient with fatty liver and a fatty liver tissue derived from a non-human animal model (hereinafter referred to as a “chimeric non-human animal”), which is obtained by transplanting human hepatocytes to an immunodeficient non-human animal with liver disorder.

[0036] Human hepatocytes can be collected from a fatty liver tissue in accordance with a method known in the art such as a collagenase perfusion method by use of a means such as a centrifuge, an elutriator, FCM (flow cytometry) and a monoclonal antibody specifically recognizing human hepatocytes. The “human hepatocytes derived from fatty liver” of the present invention are preferably human hepatocytes collected from a chimeric non-human animal, in view of large-scale production and stable supply.

[1-2] Human Hepatocytes Collected from Chimeric Non-Human Animal

[0037] The human hepatocytes collected from a fatty liver tissue derived from a chimeric non-human animal available in the present invention can be prepared in accordance with the following method.

1-2-1 Chimeric Non-Human Animal

[0038] In the present invention, the “chimeric non-human animal” refers to a non-human animal having hepatocytes of the liver partly or wholly replaced with human hepatocytes.

[0039] The “non-human animal” is preferably a mammal and more preferably a rodent. Examples of the rodent include a mouse, a rat, a guinea pig, a squirrel and a hamster. Of them, a mouse or rat generally used as an experimental animal is particularly preferable.

[0040] A chimeric non-human animal having human hepatocytes can be obtained by transplanting human hepatocytes to an immunodeficient non-human animal with a liver disorder in accordance with a method known in the art (Japanese Patent Laid-Open No. 2002-45087, WO2008/001614, WO2013/145331)

1-2-2 Immunodeficient Non-Human Animal with a Liver Disorder

[0041] The “immunodeficient non-human animal with a liver disorder” refers to an animal being immunodeficient (showing no rejection response to xenogeneic cells) and having a damage in hepatocytes derived from a non-human animal. Since hepatocytes derived from a non-human animal are damaged, human hepatocytes transplanted easily proliferate and also the function of the liver can be maintained by the human hepatocytes transplanted.

[0042] An immunodeficient non-human animal with a liver disorder can be prepared by applying a liver-disorder induction treatment and an immunodeficiency induction treatment to a same individual. Examples of the “liver-disorder induction treatment” include administration of a liver-disorder induction substance (for example, carbon tetrachloride, yellow phosphorus, D-galactosamine, 2-acetylaminofluorene, pyrrolizidine alkaloid) and a surgical treatment (for example, partial excision of the liver). Examples of the “immunodeficiency induction treatment” include administration of an immunosuppressant and excision of the thymus.

[0043] Alternatively, the immunodeficient non-human animal with a liver disorder can be prepared by applying a liver-disorder induction treatment to a genetically immunodeficient animal.

Examples of the genetically immunodeficient animal that can be used include a severe combined immunodeficient (SCID) animal showing T cell system failure, an animal losing T cell function due to genetic defect of the thymus and a RAG2 gene knockout animal. Specific examples thereof that can be used include SCID mouse, NUDE mouse, RAG2 knockout mouse, IL2Rgc/Rag2 knockout mouse, NOD mouse, NOG mouse, nude mouse, nude rat and an immunodeficient rat, which is obtained by transplanting the bone marrow of an SCID mouse to an X irradiated nude rat

(Japanese Patent Laid-Open No. 2007-228962, Transplantation, 60 (7): 740-7, 1995).

[0044] Alternatively, the immunodeficient non-human animal with a liver disorder can be prepared by applying an immunodeficiency induction treatment to an animal genetically having a liver disorder. As the animal genetically having a liver disorder, a transgenic animal, which is obtained by introducing a liver-disorder inducing protein-encoding gene ligated under control of an enhancer and/or promoter for a hepatocyte-specifically expressed protein, can be used. Examples of the “hepatocyte-specifically expressed protein” include serum albumin, cholinesterase and Hageman factor. Enhancers and/or the promoters for controlling expression of these genes can be used. Examples of the “liver-disorder inducing protein” include an urokinase plasminogen activator (uPA) and a tissue plasminogen activator (tPA). In the transgenic animal as mentioned above, since a liver-disorder inducing protein is expressed specifically to hepatocytes under control of an enhancer and/or promoter for a hepatocyte-specifically expressed protein, a liver disorder is induced. Alternatively, the animal genetically having a liver disorder can be prepared by knockout of a gene responsible for liver function. Examples of the “gene responsible for liver function” include a fumarylacetoacetate hydrase gene.

[0045] Alternatively, the immunodeficient non-human animal with a liver disorder can be prepared by crossing a genetically immunodeficient animal with an animal of the same species genetically having a liver disorder.

[0046] Alternatively, the immunodeficient non-human animal with a liver disorder can be prepared by introducing a genetic factor causing immunodeficiency and/or a liver disorder as mentioned above to a non-human animal, a fertilized egg derived from a non-human animal having genetic immunodeficiency and/or genetic liver disorder, or pluripotent stem cells (for example, embryonic stem cells (ES cells) and induced pluripotent stem cells (iPS cells)), by using a genome editing technique and a genetic engineering technique, such as gene targeting CRISPR-Cas9, zinc finger nuclease (ZFN) and TALE nuclease (TALEN) (Wang, H. et al., Cell, 153, 910-918, (2013); Yang, H. et al., Cell, 154, 1370-1379, (2013)).

[0047] In the present invention, the “immunodeficient non-human animal with a liver disorder” may have a gene specifying a trait of immunodeficiency and a gene specifying a trait of a liver disorder, each in a homozygous state or heterozygous state. As the immunodeficient non-human animal with a liver disorder of the present invention, for example, liver disorder immunodeficient mice having a genotype represented by, e.g., uPA (+/-)/SCID (+/+) and uPA (+/+)/SCID (+/+), can be suitably used.

1-2-3 Human Hepatocytes to be Transplanted

[0048] In the present invention, the “human hepatocytes” to be transplanted to an immunodeficient non-human animal with a liver disorder may be any hepatocytes as long as they are derived from a human; for example, human hepatocytes isolated from a human liver tissue by a routine method such as a collagenase perfusion method can be used. The human liver tissue may be a liver tissue derived from a healthy person or derived from a patient affected with a disease such as fatty liver and liver cancer; however, a liver tissue derived from a healthy person is preferable. The age of the person from which hepatocytes are to be isolated is not particularly limited; however, the hepatocytes are preferably isolated from a liver tissue of a child not more than 14 years old. If hepatocytes taken from a child not more than 14 years old are used, a high replacement rate with the human hepatocytes after transplantation can be attained. The hepatocytes isolated can be once frozen and thawed and then put in use.

[0049] The human hepatocytes may be proliferative human hepatocytes capable of actively proliferating in vivo. The “proliferative human hepatocytes” refer to human hepatocytes forming colonies of a single cell type as a group and proliferating in such a manner that the size of a colony is increased under in-vitro culture conditions.

[0050] The proliferation is sometimes called as “clonal proliferation”, for the reason that the cells constituting colonies belong to a same type. The number of such cells can be further increased by

subculture.

[0051] As the proliferative human hepatocytes, human small hepatocytes are mentioned (Japanese Patent Laid-Open No. H08-112092; Japan Patent No. 3266766; U.S. Pat. No. 6,004,810, Japanese Patent Laid-Open No. H10-179148; Japan Patent No. 3211941, Japanese Patent Laid-Open No. H7-274951; Japan Patent No. 3157984, Japanese Patent Laid-Open No. H9-313172; Japan Patent No. 3014322).

[0052] The human hepatocytes isolated may be directly used or further purified and then put in use. Hepatocytes can be purified in accordance with a routine method by use of a means such as a centrifuge, an elutriator, FCM and a monoclonal antibody specifically recognizing hepatocytes which proliferate while forming colonies. As the monoclonal antibody specifically recognizing human hepatocytes and proliferative human hepatocytes, those known in the art (WO2008/001614) can be used.

[0053] Examples of the human hepatocytes that can be also used include human hepatocytes isolated from a liver tissue of a chimeric non-human animal having human hepatocytes in accordance with a routine method such as a collagenase perfusion method, the human hepatocytes once frozen and thawed, human hepatocytes obtained by induction of pluripotent stem cells (for example, embryonic stem cells (ES cells) and induced pluripotent stem cells (iPS cells)), hepatic progenitor cells such as Clip cells, human hepatocytes proliferated in vitro, cryopreserved hepatocytes, hepatocytes immortalized by introduction of, e.g., a telomerase gene and a mixture of these hepatocytes and non-parenchymal cells.

1-2-4 Transplantation of Human Hepatocytes

[0054] Human hepatocytes can be transplanted to the liver of an immunodeficient non-human animal with a liver disorder via the spleen of the non-human animal or (directly) through the portal vein. The number of human hepatocytes to be transplanted can be about 1 to 2,000,000 and preferably 200,000 to 1,000,000. The gender of the immunodeficient non-human animal with a liver disorder is not particularly limited. Also, the age in days of the immunodeficient non-human animal with a liver disorder to be used for transplantation is not particularly limited; however, an animal of about 0 to 40 days after birth and preferably about 8 to 40 days after birth can be used because human hepatocytes, which are transplanted to an animal of an early age, more actively proliferate with a growth of the animal.

[0055] The animal transplanted with human hepatocytes can be raised in accordance with a routine method. For example, if the animal is raised for about 40 to 200 days after transplantation, a chimeric non-human animal having hepatocytes of the non-human animal partly or wholly replaced with human hepatocytes, can be obtained. In the liver of the chimeric non-human animal thus obtained, the symptoms of fatty liver, such as large fat droplets and hepatic steatosis, are observed (WO2008/001614).

1-2-5 Recovery of Human Hepatocytes

[0056] Human hepatocytes are collected from a chimeric non-human animal in accordance with a routine method such as a collagenase perfusion method. Human hepatocytes are preferably collected by using a chimeric non-human animal having a high content of human hepatocytes in the hepatocytes to be collected; for example, using a chimeric non-human animal having one or more of the following features. [0057] (i) 60% or more, preferably 70% or more, more preferably 80% or more, further preferably 90% or more, further more preferably 95% or more and particularly preferably 99% or more of the hepatocytes in the liver are replaced by human hepatocytes; [0058] (ii) The blood human albumin level is 0.1 mg/mL or more, preferably 0.5 mg/mL or more, more preferably 1 mg/mL or more, further preferably 5 mg/mL or more and further more preferably 10 mg/mL or more; [0059] (iii) 12 to 21 weeks, preferably 13 to 20 weeks, more preferably 14 to 19 weeks have passed after transplantation of human hepatocytes.

[0060] The human hepatocytes collected may be directly used. Alternatively, the human hepatocytes may be purified by use of a monoclonal antibody specifically recognizing human

hepatocytes or hepatocytes of a non-human animal and put in use. When the hepatocytes isolated are reacted with a human hepatocyte-specific monoclonal antibody, the cells bound to the antibody are recovered by FCM or a magnetic cell separator.

[0061] Alternatively, when the hepatocytes isolated are reacted with a monoclonal antibody specific to non-human animal hepatocytes, the cells not bound to the antibody are recovered by means of FCM or a magnetic cell separator.

[0062] In this manner, human hepatocytes can be purified and collected.

[0063] The human hepatocytes collected are further transplanted to another immunodeficient non-human animal with a liver disorder (passage transplant) in the same manner as above, and thereafter, may be collected in the same manner as above. The passage transplant can be carried out once or a plurality of times (for example, 2 to 4 times).

[1-3] Culture of Human Hepatocytes Derived from Fatty Liver

[0064] In the present invention, human hepatocytes derived from fatty liver can be cultured using a medium generally used for culturing animal cells. Examples of the medium include, but are not limited to, Dulbecco's modified eagle medium (DMEM) and Williams medium E. DMEM can be preferably used. In the medium, if necessary, further, e.g., fetal bovine serum, insulin, an epidermal growth factor, dexamethasone, a buffer, an antibiotic substance, a pH regulator, proline, ascorbic acid and nicotinamide can be appropriately added.

[0065] In the medium, dimethyl sulfoxide (DMSO) is added. DMSO can be added such that a final concentration thereof becomes 1 to 4 wt % and preferably 1 to 2 wt %; for example, 2 wt %. Due to addition of DMSO in a medium, a function of the human hepatocytes derived from fatty liver to absorb and/or secrete lipid can be enhanced to maintain accumulation of the lipid.

[0066] The human hepatocytes derived from fatty liver are seeded in a medium in an amount of 0.21 to 21.3×10^5 cells/cm² and preferably 1.07 to 3.2×10^5 cells/cm²; for example, 2.13×10^5 cells/cm². If the amount of cells is less than 0.21 cells/cm², a sufficient amount of cells serving as human fatty-liver model cells cannot be obtained, in some cases. In contrast, if the amount is more than 21.3×10^5 cells/cm², for example, growth of the cells decreases, secretion and/or accumulation amount of lipid decreases, in some cases.

[0067] The period of the culturing of human hepatocytes derived from fatty liver is not particularly limited as long as it is sufficient for the cells to secrete and/or accumulate lipid; for example, the culturing can be carried out for a sufficient period for the cells to contain fat droplets two times or more, 3 times or more, 4 times or more or 5 times or more, preferably 6 times or more, more preferably 7 times or more, further preferably 8 times or more, and further more preferably 9 times or more as large as those in the human hepatocytes derived from fatty liver, which are cultured in the same condition except that DMSO is not contained. The culture period may be, for example, more than 3 days, preferably 4 days or more, and further preferably 5 days or more. The upper limit of the culture period is not particularly limited; for example, the upper limit can be 17 days or less and preferably 13 days or less. The medium can be appropriately exchanged in the culture period.

[0068] After completion of culture, human hepatocytes secreting and/or accumulating lipid can be used as the human fatty-liver model cells.

[2-1] Secretion Amounts of Fat Droplets and Lipoprotein

[0069] The human fatty-liver model cells of the present invention are cultured hepatocytes derived from human fatty liver, which are contain a large number of fat droplets, secrete and/or accumulate a large amount of lipid, express, e.g., fatty liver related genes, and maintain the symptoms of fatty liver, similarly to the hepatocytes in human fatty liver; and more specifically, a monolayer hepatocyte culture.

[0070] The human fatty-liver model cells of the present invention contain a large number of fat droplets therein and have a high content and/or secretion amount of lipoproteins. The phrase "contain a large number of fat droplets therein" herein means that the human fatty-liver model cells of the present invention contain fat droplets 2 times or more, 3 times or more, 4 times or more or 5

times or more, preferably 6 times or more, more preferably 7 times or more, further preferably 8 times or more and further more preferably 9 times or more as large as those in the human hepatocytes derived from fatty liver cultured (for, e.g., more than 3 days, preferably 4 days or more and further preferably 5 days or more) in the same medium and in the same conditions except that DMSO is not contained. The amount of fat droplets within the cells can be quantified by staining the fat droplets within the cells in accordance with a method known in the art such as oil red O staining, followed by extracting the pigment with an organic solvent.

[0071] The “lipoprotein” is a composite particle for transporting a lipid such as cholesterol and neutral fat from an absorption/synthesis site to an application site, and having a structure consisting of a hydrophilic substance such as a phosphorus lipid, free cholesterol and apolipoprotein arranged on the outer side and a hydrophobic substance such as cholesterol and neutral fat arranged on the inner side. The “high content and/or secretion amount of lipoproteins” refers to containing lipoproteins in an amount 5 times or more, preferably 6 times or more, more preferably 7 times or more, further preferably 8 times or more and further more preferably 9 times or more as large as lipoproteins (more preferably triglyceride) contained in the human fatty-liver cells derived from fatty liver, which are cultured (for, e.g., more than 3 days, preferably 4 days or more and further preferably 5 days or more) in the same medium and in the same conditions except that DMSO is not contained, are contained in human fatty-liver model cells of the present invention or the culture supernatant thereof. The amount of lipoproteins contained in cells or culture supernatant can be measured by a method known in the art as described later.

[2-2] Content of Lipoprotein Subclass

[0072] The human fatty-liver model cells of the present invention can be characterized by the content of a lipoprotein subclass in culture supernatant.

[0073] Lipoproteins can be classified into several subclasses in accordance with difference in properties such as the size, hydration density and electrophoretic mobility of particles. In the present invention, lipoproteins can be roughly classified into 4 groups: chylomicron (CM), very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL), based on the particle sizes described below, in accordance with a method known in the art (Okazaki, The Physico-Chemical Biology (2005), Vol. 49, No. 4, p. 111-118). VLDL can be classified into 3 subclasses: Large, Medium and Small; LDL can be classified into 4 subclasses: Large, Medium, Small and Very Small; and HDL can be classified into 5 subclasses: Very Large, Large, Medium, Small and Very Small, each based on the particle size. CM can be further classified into 2 subclasses: G01 and G02; VLDL can be further classified into 5 subclasses: G03, G04, G05, G06 and G07; LDL can be further classified into 6 subclasses: G08, G09, G10, G11, G12, and G13; and HDL can be further classified into 7 subclasses: G14, G15, G16, G17, G18, G19 and G20, each based on the particle size.

TABLE-US-00001 TABLE 1 VLDL CM L M S Subclass G01 G02 G03 G04 G05 G06 G07 Particle >90 75 64 53.6 44.5 36.8 31.8 size (nm) LDL HDL L M S VS VL L M S VS Subclass G08 G09 G10 G11 G12 G13 G14 G15 G16 G17 G18 G19 G20 Particle 28.6 25.5 23 20.7 18.6 16.7 15 13.5 12.1 10.9 9.8 7.6 6.8 size (nm) CM = Chylomicron VLDL = Very Low-Density Lipoprotein LDL = Low-Density Lipoprotein HDL = High-Density Lipoprotein VL = Very Large; L = Large; M = Medium; S = Small; VS = Very Small Particle size = Peak value by HPLC method

[0074] Subclasses of lipoproteins or in culture supernatant can be quantified by fractionation by a method known in the art (WO2007/052789; Japanese Patent Laid-Open No. H9-15225; Arterioscler Thromb Vasc Biol. 2005; 25: 1-8; LipoSEARCH (registered trademark) (Skylight Biotech, Inc.)) using gel filtration liquid chromatography.

[0075] In the culture supernatant of the human fatty-liver model cells of the present invention, the content of VLDL among lipoproteins is the highest.

[0076] In the culture supernatant of the human fatty-liver model cells of the present invention, the content of VLDL is higher than that of LDL, for example, 2 times or more, 3 times or more, 4

times or more, 5 times or more, 6 times or more, 7 times or more, 8 times or more, 9 times or more, 10 times or more or 15 times or more as high as that of LDL.

[0077] More specifically, in the culture supernatant of the human fatty-liver model cells of the present invention, the content of cholesterol of VLDL is higher than that of cholesterol of LDL, for example, 2 times or more, 3 times or more, 4 times or more, 5 times or more, 6 times or more, 7 times or more, 8 times or more, 9 times or more, 10 times or more or 15 times or more as high as that of cholesterol of LDL; and the content of neutral fat (triglyceride) of VLDL is higher than that of neutral fat (triglyceride) of LDL, for example, 2 times or more, 3 times or more, 4 times or more, 5 times or more, 6 times or more, 7 times or more, 8 times or more, 9 times or more, 10 times or more or 15 times or more as high as that of neutral fat (triglyceride) of LDL.

[0078] Further, in the culture supernatant of the human fatty-liver model cells of the present invention, the content of VLDL is higher than that of HDL, for example, 5 times or more, 10 times or more, 15 times or more, 20 times or more, 25 times or more, 30 times or more, 40 times or more, 50 times or more, 60 times or more, 70 times or more, 80 times or more or 90 times or more as high as that of HDL.

[0079] More specifically, in the culture supernatant of the human fatty-liver model cells of the present invention, the content of cholesterol of VLDL is higher than that of cholesterol of HDL, for example, 5 times or more, 10 times or more, 15 times or more, 20 times or more, 25 times or more, 30 times or more, 40 times or more, 50 times or more, 60 times or more, 70 times or more, 80 times or more or 90 times or more as high as that of cholesterol of HDL; and the content of neutral fat (triglyceride) of VLDL is higher than that of neutral fat (triglyceride) of HDL, for example, 5 times or more, 10 times or more, 15 times or more, 20 times or more, 25 times or more, 30 times or more, 40 times or more, 50 times or more, 60 times or more, 70 times or more, 80 times or more or 90 times or more as high as that of neutral fat of HDL.

[0080] In the culture supernatant of the human fatty-liver model cells of the present invention, the content of LDL is higher than that of HDL, for example, 2.5 times or more, 3 times or more, 4 times or more, 5 times or more, 6 times or more, 7 times or more, 8 times or more, 9 times or more or 10 times or more as high as that of HDL.

[0081] More specifically, in the culture supernatant of the human fatty-liver model cells of the present invention, the content of cholesterol of LDL is higher than that of cholesterol of HDL, for example, 2.5 times or more, 3 times or more, 4 times or more, 5 times or more, 6 times or more, 7 times or more, 8 times or more, 9 times or more or 10 times or more as high as that of cholesterol of HDL; and the content of neutral fat (triglyceride) of LDL is higher than that of neutral fat (triglyceride) of HDL, for example, 2.5 times or more, 3 times or more, 4 times or more, 5 times or more, 6 times or more, 7 times or more, 8 times or more, 9 times or more or 10 times or more as high as that of neutral fat (triglyceride) of HDL.

[0082] In the culture supernatant of the human fatty-liver model cells of the present invention, Large-VLDL among Large-VLDL, Medium-VLDL and Small-VLDL of VLDL is contained in the highest percentage. Large-VLDL is contained in a percentage of 70 mass % or more, preferably 75 mass % or more, more preferably 80 mass % or more, further preferably 81 mass % or more, further more preferably 82 mass % or more (for example, 83 mass % or more, 84 mass % or more) and particularly preferably 85 mass % or more of the total mass of VLDL.

[0083] More specifically, in the culture supernatant of the human fatty-liver model cells of the present invention, the amount of neutral fat (triglyceride) of VLDL is the sum of the amounts of Large-VLDL, Medium-VLDL, and Small-VLDL neutral fats. Of them, a neutral fat (triglyceride) of Large-VLDL is contained in the highest percentage, 70 mass % or more, preferably 75 mass % or more, more preferably 80 mass % or more, further preferably 81 mass % or more, further more preferably 82 mass % or more (for example, 83 mass % or more, 84 mass % or more) and particularly preferably 85 mass % or more, based on the total amount of the neutral fat (triglycerides) of VLDL. In the culture supernatant of the human fatty-liver model cells of the

present invention, the amount of cholesterol of VLDL is the sum of the amounts of Large-VLDL, Medium-VLDL and Small-VLDL cholesterol. Of them, a cholesterol of Large-VLDL is contained in the highest percentage, 70 mass % or more, preferably 75 mass % or more, more preferably 80 mass % or more, further preferably 81 mass % or more, further more preferably 82 mass % or more (for example, 83 mass % or more, 84 mass % or more) and particularly preferably 85 mass % or more, based on the total amount of the cholesterol of VLDL.

[2-3] Expression Level of Fatty Liver Related Gene

[0084] The human fatty-liver model cells of the present invention can be characterized also by the expression levels of fatty liver related genes in the cells.

[0085] In the present invention, the “fatty liver related genes” refer to genes the expression of which increases in the hepatocytes of fatty liver compared in hepatocytes of healthy liver.

Examples of the fatty liver related genes include a gene encoding fatty acid synthase (gene name: FASN), a gene encoding SREBP-1 (gene name: SREBF1), a gene encoding glucose-6-phosphatase (G6PC), a gene encoding cholesterol 7 α -hydroxylase (CYP7A1), a gene encoding a cholesteryl ester transfer protein (CETP), a gene encoding glucokinase (GCK) and a gene encoding phosphoenolpyruvate carboxykinase 1 (PCK1). The “high” expression levels of fatty liver related genes means that the expression levels of fatty liver related genes are high compared to those in human hepatocytes derived from fatty liver cultured in a DMSO-free medium or in a DMSO-containing medium, for 3 days or less, for example, 2 times or more, preferably 3 times or more, more preferably 3.5 times or more as high as those.

[0086] The expression level of the fatty liver related genes can be quantified by a method known in the art, and preferably microarray analysis.

[0087] The human fatty-liver model cells of the present invention are preferably cultured in medium containing DMSO.

[0088] The human fatty-liver model cells of the present invention, since the content of VLDL among the lipoproteins is the largest, can be used as a model analogous to the hepatocytes of human fatty liver, compared to the hepatocytes (e.g., HepG2, HuH7) known in the art.

[2-4] Use of Human Fatty-Liver Model Cells

[0089] The human fatty-liver model cells of the present invention can be used as a human fatty liver model and employed in a method for screening for a substance having an effect on dyslipidemia such as fatty liver based on an increase/decrease of very low density lipoprotein (VLDL) content in the culture supernatant as an index.

[0090] The screening can be made by administering a test substance to a culture of the human fatty-liver model cells of the present invention and comparing the presence or absence of an increase/decrease of VLDL and/or a degree thereof in the culture supernatant between cells to which the test substance is administered and cells to which the test substance is not administered. More specifically, the screening can be made by administering the test substance to a culture of the human fatty-liver model cells of the present invention and comparing the presence or absence of an increase/decrease of VLDL neutral fat (triglyceride) amount or cholesterol amount and/or a degree thereof in the culture supernatant between cells to which the test substance is administered and cells to which the test substance is not administered. More preferably, the screening can be made by administering the test substance to a culture of the human fatty-liver model cells of the present invention and comparing the presence or absence of an increase/decrease of Large-VLDL and/or a degree thereof in the culture supernatant between cells to which the test substance is administered and cells to which the test substance is not administered. More specifically, the screening can be made by administering the test substance to a culture of the human fatty-liver model cells of the present invention and comparing the presence or absence of an increase/decrease of Large-VLDL neutral fat (triglyceride) amount or cholesterol amount and/or a degree thereof in the culture supernatant between cells to which the test substance is administered and cells to which the test substance is not administered. The “cells to which the test substance is administered and cells to

which the test substance is not administered” may be the same culture before and after administration of the test substance or separate cultures obtained in the same procedure except the presence or absence of the test substance.

[0091] In the present invention, “dyslipidemia” refers to fatty liver, hyperlipidemia and a disease and symptom or a disease and symptom highly likely developed from them (for example, arteriosclerosis, thrombus, NASH, liver cirrhosis, liver cancer, myocardial infarction, stroke).

[0092] In the present invention, the “substance having an effect on dyslipidemia” refers to, e.g., a substance effective for dyslipidemia or a substance effecting a negative effect in dyslipidemia. The “substance effective for dyslipidemia” refers to a substance that can decrease the production of VLDL in human fatty-liver cells. More specifically, the substance means a substance that can decrease the amount of VLDL neutral fat (triglyceride) or cholesterol produced by human fatty-liver cells. More preferably, the substance means a substance that can decrease the secretion amount of Large-VLDL in human fatty-liver cells. More specifically, the substance means a substance that can decrease the amount of large-VLDL neutral fat (triglyceride) or cholesterol produced by human fatty-liver cells. Such substances can contribute to treating or improving dyslipidemia. Usually in fatty liver, it is known that production of VLDL of lipoprotein and the amount of VLDL neutral fat (triglyceride) increase.

[0093] There is a correlation between the production of neutral fat (triglyceride) and the size of VLDL (“Introduction to Lipids and Lipoproteins” Kenneth R. et al., Endotext[Internet]. Jan. 19, 2021). It has been confirmed that as the production of neutral fat (triglyceride) increases, the amount of Large-VLDL increases. Because of this, in the culture supernatant of the human fatty-liver model cells of the present invention to which a test substance is administered, if a reduction in VLDL amount (particularly, VLDL neutral fat (triglyceride) amount or cholesterol amount), preferably Large-VLDL amount (particularly, Large-VLDL neutral fat (triglyceride amount) or cholesterol amount) is confirmed, the test substance can be determined as a substance that improves dyslipidemia, particularly at least one of the symptoms of fatty liver, that is, a “substance effective for dyslipidemia” of the present invention. Large-VLDL is metabolized to produce Small-LDL (Ronald M Krauss, Diabetes Care. 2004 June; 27 (6): 1496-504). Small-LDL is easily denatured by oxidation and deposited on the wall of blood vessels and may cause arteriosclerosis (Kiniwa et al., The Journal of Japanese College of Angiology, Vol. 46, 2006, p. 441-448). Because of this, in the culture supernatant of the human fatty-liver model cells of the present invention to which a test substance is administered, if a reduction in VLDL amount (particularly, VLDL neutral fat (triglyceride) amount or cholesterol amount), preferably Large-VLDL amount (particularly, Large-VLDL neutral fat (triglyceride) amount or cholesterol amount) is confirmed, the test substance can be determined as a substance that improves dyslipidemia, particularly at least one of the symptoms of arteriosclerosis, that is, a “substance effective for dyslipidemia” of the present invention. Specific examples of such a substance that reduces the VLDL amount include substances known as active ingredients for hyperlipidemia drugs, such as fenofibrate and lomitapide. As more specifically described in the following Examples, the substance can be detected by using the human fatty-liver model cells of the present invention based on an increase/decrease of VLDL in the culture supernatant as an index. A substance effective for diseases and symptoms of dyslipidemia is determined as effective for not only treatment or improvement but also prevention of diseases and symptoms thereof.

[0094] The “substance effecting a negative effect in dyslipidemia” refers to a substance likely to exacerbate at least one symptom of human fatty-liver cells and, in the present invention, refers to a substance that can increase the production of VLDL in human fatty-liver cells. More specifically, the substance means a substance that can increase the amount of VLDL (particularly, VLDL neutral fat (triglyceride) amount or cholesterol amount) produced by human fatty-liver cells, preferably the amount of Large-VLDL (particularly, Large-VLDL neutral fat (triglyceride) amount or cholesterol amount). As mentioned above, there is a correlation between dyslipidemia such as fatty liver and

arteriosclerosis and VLDL amount (particularly, VLDL neutral fat (triglyceride) amount or cholesterol amount), and preferably Large-VLDL amount (particularly, Large-VLDL neutral fat (triglyceride) amount or cholesterol amount)). Because of this, in the culture supernatant of the human fatty-liver model cells of the present invention to which a test substance is administered, if an increase in VLDL amount (particularly, VLDL neutral fat (triglyceride) amount or cholesterol amount), preferably Large-VLDL amount (particularly, Large-VLDL neutral fat (triglyceride) amount or cholesterol amount) is confirmed, the test substance is determined as a substance that likely exacerbates at least one of the symptoms of dyslipidemia such as fatty liver and arteriosclerosis, that is, a “substance effecting a negative effect in dyslipidemia” of the present invention. Examples of such a substance that increases VLDL amount include oleic acid. As more specifically described in the following Examples, the substance can be detected by using the human fatty-liver model cells of the present invention based on an increase/decrease of VLDL in the culture supernatant as an index.

[0095] Examples of the test substance include, but are not limited to, a small molecule compound, an amino acid, a nucleic acid, lipid, sugar and an extract of a natural product.

[0096] Now, the present invention will be more specifically described by way of Examples; however, the present invention is not limited to these.

EXAMPLES

I. Test Method

1. Preparation of Human Hepatocytes Derived from Fatty Liver

(Preparation of Chimeric Mice (PXB Mice) Having Human Hepatocytes)

[0097] PXB mice were prepared in accordance with a method known in the art (Japanese Patent Laid-Open No. 2002-45087). More specifically, a mouse, which had liver damage given in accordance with a genetic engineering procedure by introducing an urokinase plasminogen activator (uPA) gene ligated to an enhancer and a promoter of albumin to be synthesized in the liver (cDNA-uPA) to all cells, was crossed with an immunodeficient mouse (SCID mouse) to prepare immunodeficient mice with liver disorder (cDNA-uPA (+/-)/SCID mice).

[0098] The mice (cDNA-uPA (+/-)/SCID) of 3 weeks old were anesthetized. Skin around the spleen and rectus abdominis were cut by scissors. The tip of the spleen was picked up and fixed at the position to facilitate introduction of cells. Subsequently, using a glass syringe filled with a human hepatocyte suspension, human hepatocytes were injected from the tip of the spleen by inserting the needle. Thereafter, the spleen was returned to the mice and the skin and peritoneum were sawed by use of a plastic surgery needle to close the incision site. After confirming no abnormality of breathing of the mice transplanted, the mice were raised in a rearing cage.

[0099] PXB mice of 17 to 22 weeks old, which had a body weight of 15 to 20 g and a serum human albumin content of 10 mg/mL or more (replacement rate of human hepatocytes calculated based on the amount of human albumin was 95% or more), were selected and used in the following experiments.

(Separation of Cells)

[0100] The PXB mouse under anesthesia was placed on a dissection table, fixed with medical tape, and then, subjected to laparotomy. An intravenous cannula was inserted in the portal vein, perfusate A was fed to remove the blood. Perfusate B was fed to dissolve collagen in the liver tissue and the liver is excised out so as not to damage the intestinal tract and stomach. The liver was shaken in perfusate C to release/separate hepatocytes. Undigested tissue pieces were removed by passing the resultant solution through a cell strainer and the hepatocytes were recovered in a tube.

(Preparation of Cells)

[0101] The hepatocytes (PXB-cells) recovered were centrifuged. After the supernatant was removed, 40 mL of medium A was added to the resultant sediment. The mixture was gently stirred. This operation was repeated twice to remove, e.g., impurities and lipid suspended in the supernatant. The resultant solution was passed through a cell strainer to isolate and collect cell

mass in a tube. The number of cells was counted in accordance with a trypan blue dye exclusion method by a hemocytometer. Based on the count value, the density, total number and survival rate of cells were obtained.

(Seeding)

[0102] Based on the cell density of the cell suspension, the dilution rate for obtaining a desired seeding density was calculated and the cell suspension was diluted with medium A. To each of the wells of a culture plate, the diluted cell suspension (500 μ L) was gently poured. The plate was allowed to stand still for about 20 minutes until the cells were slightly in contact with the bottom surface of the wells and gently placed in an incubator (37° C., 5% CO.sub.2) to culture the cells.

2. Analysis of Lipoprotein in the Culture Supernatant of Cells Cultured in DMSO-Containing Medium

[0103] The following day of seeding, medium A was removed. Instead, 500 μ L of medium B (DMSO (+)) or medium C (DMSO (-)) was added. The plate was gently placed in an incubator (37° C., 5% CO.sub.2) and the cells were cultured for 5 days. After completion of culture, an image of the cells was taken by a photomicrographic camera. Then, the culture supernatant was recovered and subjected to analysis for lipoprotein in the culture supernatant described below.

3. Analysis of Lipoprotein in the Culture Supernatant of Cells Cultured in DMSO-Containing Medium for 5 to 14 Days

Culture in DMSO-Containing Medium for 5 Days The following day of seeding, medium A was removed.

[0104] Instead, 500 μ L of medium B (DMSO (+)) was added. The plate was gently placed in an incubator (37° C., 5% CO.sub.2) and the cells were cultured. Day 1 and Day 2 after initiation of culture with medium B (DMSO (+)), the medium was exchanged with fresh medium B (DMSO (+)).

[0105] After completion of culture with medium B (DMSO (+)) for 5 days, the following analysis for lipoprotein in the culture supernatant was carried out.

Culture in DMSO-Containing Medium for 9 Days

[0106] The following day of seeding, medium A was removed. Instead, 500 μ L of medium B (DMSO (+)) was added. The plate was gently placed in an incubator (37° C., 5% CO.sub.2) and the cells were cultured. Day 5, Day 7 and Day 8 after initiation of culture with medium B (DMSO (+)), the medium was exchanged with fresh medium B (DMSO (+)).

[0107] After completion of culture with medium B (DMSO (+)) for 9 days, the following analysis for lipoprotein in the culture supernatant was carried out.

Culture in DMSO-Containing Medium for 12 Days

[0108] The following day of seeding, medium A was removed. Instead, 500 μ L of medium B (DMSO (+)) was added. The plate was gently placed in an incubator (37° C., 5% CO.sub.2) and the cells were cultured. Day 5, Day 7 and Day 8 after initiation of culture with medium B (DMSO (+)), the medium was exchanged with fresh medium B (DMSO (+)).

[0109] After completion of culture with medium B (DMSO (+)) for 12 days, the following analysis for lipoprotein in the culture supernatant was carried out.

Culture in DMSO-Containing Medium for 14 Days

[0110] The following day of seeding, medium A was removed. Instead, 500 μ L of medium B (DMSO (+)) was added. The plate was gently placed in an incubator (37° C., 5% CO.sub.2) and the cells were cultured. Day 5, Day 7, Day 8 and Day 12 after initiation of culture with medium B (DMSO (+)), the medium was exchanged with fresh medium B (DMSO (+)).

[0111] After completion of culture with medium B (DMSO (+)) for 14 days, the following analysis for lipoprotein in the culture supernatant was carried out.

4. Analysis of Lipoprotein in the Cells Cultured in DMSO-Containing Medium for 5 to 14 Days

Culture in DMSO-Containing Medium for 5 Days

[0112] The following day of seeding, medium A was removed. Instead, 500 μ L of medium B

(DMSO (+)) was added. The plate was gently placed in an incubator (37° C., 5% CO.sub.2) and the cells were cultured. Day 1 and Day 2 after initiation of culture with medium B (DMSO (+)), the medium was exchanged with fresh medium B (DMSO (+)).

[0113] After completion of culture with medium B (DMSO (+)) for 5 days, the following analysis for intracellular lipoproteins, more specifically, the amounts of cholesterol and triglyceride in the cells, was carried out.

Culture in DMSO-Containing Medium for 9 Days

[0114] The following day of seeding, medium A was removed. Instead, 500 μ L of medium B (DMSO (+)) was added. The plate was gently placed in an incubator (37° C., 5% CO.sub.2) and the cells were cultured. Day 5, Day 7 and Day 8 after initiation of culture with medium B (DMSO (+)), the medium was exchanged with fresh medium B (DMSO (+)).

[0115] After completion of culture with medium B (DMSO (+)) for 9 days, the following analysis for intracellular contents of lipoproteins, more specifically, cholesterol and triglyceride, was carried out.

Culture in DMSO-Containing Medium for 12 Days

[0116] The following day of seeding, medium A was removed. Instead, 500 μ L of medium B (DMSO (+)) was added. The plate was gently placed in an incubator (37° C., 5% CO.sub.2) and the cells were cultured. Day 5, Day 7 and Day 8 after initiation of culture with medium B (DMSO (+)), the medium was exchanged with fresh medium B (DMSO (+)).

[0117] After completion of culture with medium B (DMSO (+)) for 12 days, the following analysis for intracellular contents of lipoproteins, more specifically, cholesterol and triglyceride, was carried out.

Culture in DMSO-Containing Medium for 14 Days

[0118] The following day of seeding, medium A was removed. Instead, 500 μ L of medium B (DMSO (+)) was added. The plate was gently placed in an incubator (37° C., 5% CO.sub.2) and the cells were cultured. Day 5, Day 7, Day 8 and Day 12 after initiation of culture with medium B (DMSO (+)), the medium was exchanged with fresh medium B (DMSO (+)).

[0119] After completion of culture with medium B (DMSO (+)) for 14 days, the following analysis for intracellular contents of lipoproteins, more specifically, cholesterol and triglyceride, was carried out.

Culture of Hepatocytes Known in the Art

[0120] Hepatocytes (HepG2 cells, HuH7 cells) known in the art were seeded in 500 μ L of medium A, gently placed in an incubator (37° C., 5% CO.sub.2) and cultured for 7 days.

[0121] After completion of culture, the following analyses for lipoproteins in the culture supernatant and within the cells, were carried out.

5. Analysis Method

(Analysis of Lipoprotein in Culture Supernatant)

[0122] Lipoproteins contained in the culture supernatants of PXB-cells, HepG2 cells and HuH7 cells were analyzed by using LipoSEARCH (registered trademark) method (Skylight Biotech, Inc.).

[0123] After completion of the culture mentioned above, the medium used in the culture was removed and 500 μ L of medium D for analysis was added. The culture plate was gently placed in an incubator (37° C., 5% CO.sub.2) and the cells were cultured for 2 days. Thereafter, the culture supernatant was recovered, lipoproteins contained in the culture supernatant (80 μ L) were fractionated into 4 types of subgroups, CM, VLDL, LDL and HDL fractions, by gel filtration HPLC. Cholesterol and neutral fat (triglyceride) contained in individual fractions were quantified by online enzyme reactions. Concentration analysis was carried out in accordance with the computer program specifically developed by Skylight Biotech, Inc. Note that, in the enzymatic reaction, Diacolor Liquid TG-S (manufactured by Toyobo Co., Ltd.) was used. As standard sera for cholesterol and neutral fat (triglyceride) concentration in CM, VLDL, LDL and HDL, the sera manufactured by Kyowa Hakko Kirin Co., Ltd. were used.

(Analysis for Lipoprotein in Cells)

Measurement of Triglyceride in Cells

[0124] Triglyceride in cells was measured by use of Cholestest (registered trademark) TG (Sekisui Medical Co., Ltd.) in accordance with the instruction by the manufacturer. More specifically, the cells were washed with PBS, and then, completely dewatered (stored at -80°C . until measurement). To each well containing the cells, 200 μL of TG enzyme solution (1) was added. A reaction was allowed to proceed while keeping the cells warm at 37°C . for 10 minutes (free glycerol was removed) Then, the cells were torn away by pipetting, transferred to a centrifuge tube and centrifuged at $10,000\text{ rpm}\times 10\text{ minutes}$. Then, the supernatant (7.5 μL) was transferred to a 96-well microplate. To this, 68 μL of TG enzyme solution (1) was added and the microplate was kept warm at 37°C . for 10 minutes to remove completely free glycerol. Then, 25 μL of TG enzyme solution (2) was added and kept the plate warm at 37°C . for 10 minutes. The resultant reaction product was subjected to measurement of absorbance at 550 nm. The content of triglyceride was calculated based on HDL-C180A as the reference (triglyceride concentration of HDL-C180A was 52.26 mg/dL).

Measurement of Intracellular Cholesterol

[0125] Intracellular cholesterol was measured by use of Cholestest (registered trademark) CHO (Sekisui Medical Co., Ltd.) in accordance with the instruction by the manufacturer. More specifically, the cells were washed with PBS, and then, completely dewatered (stored at -80°C . until measurement). To each well containing the cells, 200 μL of CHO enzyme solution (1) was added and kept the cells warm at 37°C . for 10 minutes. Then, the cells were torn away by pipetting, transferred to a centrifuge tube and centrifuged at $10,000\text{ rpm}\times 10\text{ minutes}$. Then, the supernatant (15 μL) was transferred to a 96-well microplate. To this, 68 μL of CHO enzyme solution (1) was added and kept warm at 37°C . for 10 minutes. Subsequently, 25 μL of CHO enzyme solution (2) was added to the wells and kept the plate warm at 37°C . for 10 minutes. The resultant reaction product was subjected to measurement of absorbance at 550 nm. The content of cholesterol was calculated based on HDL-C180A as the standard (triglyceride concentration of HDL-C180A was 152.67 mg/dL).

6. Analysis for Gene Expression Level

[0126] The total RNA of PXB-cells cultured in medium B for 3 days or 6 days was extracted by use of TRIzol (registered trademark)+Direct zol (Thermo Fisher Scientific k.k.) in accordance with the instruction by the manufacturer.

[0127] After the quality of the total extracted RNA sample was evaluated by a bioanalyzer (Agilent Technologies, Inc.), microarray analysis (Agilent Technologies, Inc.) was carried out in accordance with the instruction by the manufacturer to analyze the expression levels of fatty liver related genes, FASN, SREBF1, G6PC, CYP7A1, CETP, GCK and PCK1.

7. Perfusate, Medium

[0128] The compositions of perfusate A, perfusate B, perfusate C, medium A, medium B, medium C and medium D used herein are as follows.

TABLE-US-00002 TABLE 2 Name of reagent Final concentration
Perfusate A HBSS(−) — D-glucose 1 mg/mL EGTA 200 $\mu\text{g/mL}$ 50 mg/mL Gentamycin 10 $\mu\text{g/mL}$ 1M HEPES buffer 10 mM
Perfusate B HBSS(−) — Type IV collagenase 0.05% CaCl₂ 600 $\mu\text{g/mL}$ 50 mg/mL
Gentamycin 10 $\mu\text{g/mL}$ 1M HEPES buffer 10 mM Trypsin inhibitor 100 $\mu\text{g/mL}$
Perfusate C HBSS(−) — 10% Albumin solution 10 mg/mL 50 mg/mL Gentamycin 10 $\mu\text{g/mL}$ 1M HEPES buffer 10 mM

TABLE-US-00003 TABLE 3 Medium A Reagent Final concentration
Dulbecco's modified eagle medium — NaHCO₃ 44 mM Penicillin G 100 IU/mL Streptomycin 100 $\mu\text{g/mL}$ N-2-Hydroxyethylpiperazine-N-2- 20 mM ethanesulfonic acid (HEPES) Fetal bovine serum (FBS) 10%

TABLE-US-00004 TABLE 4 Reagent Final concentration
Medium B Dulbecco's modified eagle medium — NaHCO₃ 44 mM Penicillin G 100 IU/mL Streptomycin 100 $\mu\text{g/mL}$ HEPES 20

mM FBS 10% L-Proline 15 µg/mL Insulin 0.25 µg/mL Dexamethasone 50 nM Epidermal growth factor (EGF) 5 ng/mL L-Ascorbic acid 2-phosphate 0.1 mM (Asc-2P) Dimethyl sulfoxide (DMSO) 2% Medium C (Medium B minus DMSO) Dulbecco's modified eagle medium — NaHCO₃ 44 mM Penicillin G 100 IU/mL Streptomycin 100 µg/mL HEPES 20 mM FBS 10% L-Proline 15 µg/mL Insulin 0.25 µg/mL Dexamethasone 50 nM EGF 5 ng/mL Asc-2P 0.1 mM Medium D Williams medium E — CM4000 Concentration recommended by manufacturer

II. Results

1. Analysis Results of Lipoprotein Contained in the Culture Supernatant of Cells Cultured in DMSO-Containing Medium

[0129] FIG. 1 shows PXB-cells cultured in medium B (DMSO (+)) or medium C (DMSO (-)) for 5 days. In the PXB-cells cultured in medium B (DMSO (+)) (FIG. 1, left) compared to PXB cells cultured in medium C (DMSO (-)) (FIG. 1, right), a large number (about double) of fat droplets (white droplets) were observed. In any mediums, PXB-cells formed a monolayer cell culture.

[0130] FIG. 2 shows the measurement results of the content of total neutral fat (triglyceride) of lipoproteins (including CM, VLDL, LDL and HDL) contained in the culture supernatants recovered after culture in each of medium B (DMSO (+)) and medium C (DMSO (-)) for 5 days. The results are shown by relative values based on the content (regarded as “100”) of the total neutral fat in the culture supernatant of PXB-cells cultured in medium B (DMSO (+)). It was confirmed that secretion of the total neutral fat (triglyceride) in lipoproteins contained in PXB-cells cultured in medium B (DMSO (+)) is about 9 times as high as that cultured in medium C (DMSO (-)).

[0131] From the results, it was confirmed that if PXB-cells are cultured in a DMSO-containing medium, accumulation/secretion of lipid can be kept at a high level.

2. Analysis Results of Lipoprotein Contained in the Culture Supernatant of Cells Cultured in DMSO-Containing Medium for 5 to 14 Days

[0132] FIG. 3 shows analysis results of lipoproteins in the culture supernatants of PXB-cells in medium B (DMSO (+)) for 5, 9, 12 and 14 days, and hepatocytes known in the art (HepG2 cells, HuH7 cells).

[0133] In PXB-cells, the total cholesterol and total neutral fat (triglyceride) contents in the culture supernatant were both the highest in the case of culturing the cells for 5 days. In the cases of culturing the cells for 9 days, 12 days and 14 days, the contents thereof were maintained, albeit slightly lower.

[0134] In contrast, it was confirmed that the total cholesterol and total neutral fat (triglyceride) contents in the culture supernatant of HepG2 cells are both significantly low compared to those in the culture supernatant of PXB-cells. It was also confirmed that the total cholesterol content in the culture supernatant of HuH7 cells is equivalent to those of PXB-cells cultured for 12 days and 14 days; however, the content of the total neutral fat (triglyceride) is remarkably low compared to that of PXB-cells.

[0135] FIG. 4 shows the analysis results of lipoproteins (4 types of subgroups) contained in the culture supernatant. In the PXB-cells cultured in medium B (DMSO (+)), it was confirmed that the content of VLDL is the largest in each of the cholesterol and neutral fat (triglyceride), regardless of the culture period. The analysis results of lipoproteins (content ratio (weight ratio) of CM, VLDL, LDL, HDL) in the culture supernatant of PXB-cells cultured in medium B (DMSO (+)) for 5 days, 9 days, 12 days and 14 days will be described below.

Culture for 5 Days

(Cholesterol)

[0136] CM:VLDL:LDL:HDL=3:86:8:3

(Neutral Fat (Triglyceride))

[0137] CM:VLDL:LDL:HDL=3:91:5:1

Culture for 9 Days

(Cholesterol)

[0138] CM:VLDL:LDL:HDL=4:82:9:2

(Neutral Fat (Triglyceride))

[0139] CM:VLDL:LDL:HDL=3:90:6:1

Culture for 12 Days

(Cholesterol)

[0140] CM:VLDL:LDL:HDL=2:80:9:3

(Neutral Fat (Triglyceride))

[0141] CM:VLDL:LDL:HDL=3:90:6:1

Culture for 14 Days

(Cholesterol)

[0142] CM:VLDL:LDL:HDL=1:79:14:6

(Neutral Fat (Triglyceride))

[0143] CM:VLDL:LDL:HDL=2:91:6:1

[0144] In contrast, as to HuH7 cells, the content of LDL was the highest in each of cholesterol and neutral fat (triglyceride) contained in the culture supernatant thereof. As to HepG2 cells cultured in the same condition, the content of HDL was the highest in each of cholesterol and neutral fat (triglyceride) contained in the culture supernatant thereof.

[0145] The peak of VLDL was observed in PXB-cells and not observed in culture supernatants of HepG2 cells and HuH7 cells. It was confirmed that VLDL is secreted specifically in PXB-cells cultured in medium B (DMSO (+)).

[0146] With respect to the VLDL contained in the culture supernatant, the amount of neutral fat (triglyceride) contained in Large-VLDL, Medium-VLDL, and Small-VLDL were analyzed. The results are shown in Table 5. More specifically, the table shows the analysis results of each of culture supernatants of PXB-cells cultured in medium B (DMSO (+)) for 13 days, and thereafter in medium D for 2 days; fresh human hepatocytes (FHH) isolated from a healthy person (no pre-existing disease) and cultured in Incubation Medium (Cat No. MIL214-100 M, company: BIOPREDIC International) for one day and thereafter in medium D (company: BIOPREDIC International) for 2 days; and, HepG2 cells and HuH7 cells cultured in medium A for 2 days, and thereafter in medium D for 2 days.

[0147] The ratio of Large-VLDL to total VLDLs contained in the culture supernatant was 81.5% (40.9/50.2) in PXB-cells, 69.2% (78.6/113.5) in FHH, 0% in HepG2 cells, and 1.9% in HuH7 cells. The PXB-cells cultured in medium B (DMSO (+)) exhibited a significantly high content ratio of Large-VLDL.

TABLE-US-00005 TABLE 5 Neutral fat (triglyceride) ($\mu\text{g}/10.\text{sup.6}$ cells) PXB FHH HepG2 HuH-7 Culture 53.8 \pm 1.0 123.8 \pm 8.3 1.2 12.1 \pm 0.2 supernatant VLDL 50.2 \pm 1.0 113.5 \pm 8.1 0.1 5.3 \pm 0.2 Large-VLDL 40.9 \pm 1.0 78.6 \pm 6.8 0.0 0.1 Large- 81.5% 69.2% 0% 1.9% VLDL/VLDL(%) (40.9/50.2) (78.6/113.5) (0.1/5.3) Medium-VLDL 7.8 \pm 0.2 31.0 \pm 1.2 0.0 2.4 \pm 0.1 Small-VLDL 1.5 4.9 \pm 0.2 0.1 2.7 \pm 0.1

3. Analysis Results of Lipoprotein in the Cells Cultured in DMSO-Containing Medium for 5 to 14 Days

[0148] FIG. 5 shows the analysis results of lipoproteins in each of PXB-cells cultured in medium B (DMSO (+)) for 5, 9, 12 and 14 days and hepatocytes (HepG2 cells, HuH7 cells) known in the art.

[0149] In PXB-cells, the content of the total cholesterol was the lowest after culture for 5 days and slightly higher after culture for 9 days, 12 days and 14 days. The content of the total neutral fat (triglyceride) was the highest after culture for 5 days and maintained, albeit slightly lower after culture for 19 days, 12 days and 14 days.

[0150] In contrast, it was confirmed that the total cholesterol and total neutral fat (triglyceride) contents in HepG2 cells are both remarkably low compared to those in PXB-cells. It was also confirmed that the total neutral fat (triglyceride) content in HuH7 cells is equivalent to those of PXB-cells after culture for 9 days, 12 days and 14 days but is remarkably lower than that of PXB-

cells after culture for 5 days. It was confirmed that the total cholesterol is equivalent to those of the all periods of PXB-cells.

[0151] From these results, it was confirmed that PXB-cells, which were cultured in medium B (DMSO (+)), can maintain accumulation/secretion of lipid (cholesterol and neutral fat (triglyceride)) at least about two weeks.

[0152] Particularly, in the culture for 6 days, the highest accumulation of neutral fat (triglyceride) and secretion of cholesterol and neutral fat (triglyceride) were confirmed. It was further confirmed that the secreted lipoproteins contain VLDL in the largest amount. Such feature is not observed in hepatocytes (HuH7 cells, HepG2 cells) previously known in the art. It was demonstrated that PXB-cells cultured in the medium B (DMSO (+)) have different properties from those of hepatocytes previously known in the art.

4. Expression Level of the Fatty Liver Related Gene

[0153] FIG. 6 shows the analysis results of expression levels of fatty liver related genes (FASN, SREBF1, G6PC) in PXB-cells cultured in medium B (DMSO (+)) for 3 days and 6 days. The results are shown by relative values based on the expression level (regarded as “1”) of each gene on Day 3 after initiation of culture. In any one of the genes, the expression level on Day 6 after initiation of culture was higher than that of Day 3 after initiation of culture. Although not shown in FIG. 6, the expression levels of CYP7A1, CETP, GCK and PCK1 on Day 6 after initiation of culture similarly higher than those of Day 3 after initiation of culture.

[0154] From the above results, it was confirmed that accumulation/secretion of lipid can be maintained in PXB-cells cultured in medium B (DMSO (+)) and also fatty liver related genes are expressed. From this, it was demonstrated that PXB-cells can be used as human fatty-liver model cells.

III. Screening for Substance Having an Effect on Human Fatty Liver Based on Large-VLDL as an Index

(1-1) Addition of Oleic Acid

Preparation of Cells

[0155] PXB-cells were diluted with medium A. The diluted cell suspension (500 μ L) was gently poured to individual wells of a culture plate. The plate was allowed to stand still for about 20 minutes until the cells were slightly in contact with the bottom surface of the wells and gently placed to an incubator (37° C., 5% CO.sub.2) to culture the cells. The following day of seeding, medium A was removed and 500 μ L of medium B (DMSO (+)) was added. The plate was gently placed in an incubator (37° C., 5% CO.sub.2) and the cells were cultured for 13 days. After completion of culture, the cells were used in the following test.

Screening Test

[0156] As a test substance, oleic acid, which is known to facilitate the accumulation of lipids in hepatocytes, was used.

[0157] A medium of PXB-cells prepared above was exchanged with medium D containing 0.75 mM oleic acid, and gently placed in an incubator (37° C., 5% CO.sub.2). PXB-cells were cultured for two days.

[0158] After completion of culture, the total cholesterol and total neutral fat (triglyceride) in culture supernatant and in cells were measured in accordance with the methods described in the sections “Analysis of lipoprotein in culture supernatant” and “Analysis of lipoprotein in cells”.

[0159] As a control, medium D (5 μ L) alone was added in place of oleic acid.

(1-2) Results of Oleic Acid Addition

[0160] FIG. 7 (A) shows the analysis results of neutral fat (triglyceride) content in each of subgroups of lipoprotein in the culture supernatant of PXB-cells cultured in the presence of oleic acid for 2 days. FIG. 7 (B) shows the analysis results on the contents of neutral fat (triglyceride) of Large, Medium and Small VLDLs different in size, with respect to VLDL in the culture supernatant.

[0161] It was confirmed that the VLDL content in the culture supernatant significantly increased by the addition of oleic acid. It was confirmed that the content of Large-VLDL alone among the VLDL significantly increased.

[0162] In addition, it was confirmed that the content of neutral fat (triglyceride) in the cells to which oleic acid was added, significantly increased (data not shown). It was also confirmed that the contents of cholesterol in the culture supernatant and cells change similarly to the content of neutral fat (triglyceride) (data not shown).

(2-1) Addition of Lomitapide

Preparation of Cells

[0163] PXB-cells were prepared in the same manner as in the method set forth in “Preparation of cells” in the above step (1-1) except that the period of culture in medium B (DMSO (+)) was set at 6 days and subjected to the following test.

Screening Test

[0164] As a test substance, lomitapide (Tokyo Chemical Industry Co., Ltd.) known as a hyperlipidemia drug was used.

[0165] The mediums for the PXB-cells prepared above were exchanged with mediums D containing 1 nM or 5 nM lomitapide respectively and gently placed in an incubator (37° C., 5% CO.sub.2). PXB-cells were cultured for two days.

[0166] After completion of the culture, cholesterol and neutral fat (triglyceride) in the culture supernatant and cells were measured in accordance with the method described in the above sections “analysis of lipoprotein in culture supernatant” and “analysis of lipoprotein in cells”, respectively.

[0167] As a control, ethanol (5 μ L) alone was added in place of lomitapide.

(2-2) Results of Lomitapide Addition

[0168] FIG. 8 (A) shows the analysis results of neutral fat (triglyceride) content in each of subgroups of lipoprotein in the culture supernatant of PXB-cells cultured in the presence of lomitapide for 2 days. FIG. 8 (B) shows the analysis results of the contents of neutral fat (triglyceride) of Large, Medium and Small VLDLs different in size, with respect to VLDL in the culture supernatant.

[0169] It was confirmed that the VLDL content of the culture supernatant significantly decreases by the addition of 5 nM lomitapide. It was confirmed that the content of Large-VLDL alone among the VLDL significantly decreased.

[0170] In contrast, no change was confirmed in the content of neutral fat (triglyceride) in the cells to which lomitapide was added (data not shown). It was also confirmed that the contents of cholesterol in the culture supernatant and cells show similar results as the content of neutral fat (triglyceride) (data not shown).

(3-1) Addition of Fenofibrate

Preparation of Cells

[0171] PXB-cells were prepared in the same manner as in the method set forth in “Preparation of cells” in the above step (1-1) and subjected to the following test.

Screening Test

[0172] As a test substance, fenofibrate (Sigma-Aldrich) known as a hyperlipidemia drug was used.

[0173] The mediums of the PXB-cells prepared above were exchanged with mediums D containing 10 μ M, 50 μ M or 100 μ M fenofibrate and gently placed in an incubator (37° C., 5% CO.sub.2). The PXB-cells were cultured for 2 days. After completion of the culture, cholesterol and neutral fat (triglyceride) in the culture supernatant and cells were measured in accordance with the method described in the above section “Analysis of lipoprotein in culture supernatant” and “Analysis of lipoprotein in cells”, respectively.

[0174] As a control, ethanol (5 μ L) alone was added in place of fenofibrate.

(3-2) Results of Fenofibrate Addition

[0175] FIG. 9 (A) shows the analysis results of neutral fat (triglyceride) content in each of

subgroups of lipoprotein in the culture supernatant of PXB-cells cultured in the presence of fenofibrate for 2 days. FIG. 9 (B) shows the analysis results on the contents of neutral fat (triglyceride) of Large, Medium and Small VLDLs different in size, with respect to VLDL in the culture supernatant.

[0176] It was confirmed that the VLDL content of the culture supernatant significantly decreased by addition of each of 0 μ M, 50 μ M and 100 μ M fenofibrate. It was also confirmed that the content of any one of Large-VLDL Medium-VLDL and Small-VLDL significantly decreased.

[0177] In contrast, no change was confirmed in the content of neutral fat (triglyceride) in the cells to which fenofibrate was added (data not shown). It was also confirmed that the contents of cholesterol in the culture supernatant and cells show similar results as the content of neutral fat (triglyceride) (data not shown).

[0178] From the above results, it was demonstrated that human hepatocytes derived from fatty liver and prepared by culturing in a DMSO-containing medium can be used for screening for a substance (e.g., oleic acid) effecting a negative effect in dyslipidemia and a substance (e.g., lomitapide, fenofibrate) effective for prevention, treatment or improvement of dyslipidemia, such as a lipid metabolism improver and a hyperlipidemia drug, based on an increase/decrease of the content of VLDL in the culture supernatant, particularly Large-VLDL (particularly the content of neutral fat (triglyceride)) as an index.

Claims

1. Human fatty-liver model cells for use in a method for screening for a substance having an effect on dyslipidemia based on an increase/decrease of very low density lipoprotein (VLDL) in culture supernatant as an index, wherein the culture supernatant of the cells contains VLDL and low density lipoprotein (LDL), wherein the VLDL is contained more than the LDL.
 2. The cells according to claim 1, which is a monolayer cell culture.
 3. The cells according to claim 1 or 2, wherein the VLDL includes Large-VLDL, Medium-VLDL and Small-VLDL, and the Large-VLDL is contained in a ratio of 70 mass % or more of total VLDLs.
 4. The cells according to any one of claims 1 to 3, which are obtained by culturing human hepatocytes derived from fatty liver in a medium containing dimethyl sulfoxide.
 5. The cells according to claim 4, wherein the human hepatocytes derived from fatty liver are collected from a chimeric non-human animal having human hepatocytes.
 6. A method for screening for a substance having an effect on dyslipidemia, comprising the steps of: administering a test substance to the cells according to any one of claims 1 to 5; and comparing an amount of very low density lipoprotein (VLDL) in culture supernatant between the cells to which the test substance is administered and cells to which the test substance is not administered.
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