

DESCRIPTION

A method for the evaluation of hair growth promoting activity

Technical Field

This invention relates to a method for the evaluation of hair growth promoting activity using an antibody which is specific for epithelial new follicle, and a kit for the evaluation of hair growth promoting activity comprising said antibody.

Background Art

The normal morphogenesis of epithelial tissue has been suggested to be controlled by factors derived from mesenchymal cells present around the epithelial tissue. Diseases resulting from the abnormal morphogenesis of epithelial tissue are largely caused by abnormalities of mesenchymal cells. Therefore, an interest has arisen in the clarification of the mechanism in which mesenchymal cells controls the morphogenesis of epithelial tissue. However, substances involved in the control of morphogenesis of epithelial tissue are expressed under time and spatial control in a complicated system, and accordingly, it is extremely difficult to isolate these substances and analyze their functions. It is also difficult to construct a model experimental system simplifying the morphogenesis of epithelial tissue. For these reasons, no significant progress has been made to date in researches in this field. Thus, analysis of the controlling mechanism for the morphogenesis of epithelial tissue has been highly desired in order to elucidate the mechanism of occurrence of diseases associated with the morphogenesis of epithelial tissue and establish methods for treating these diseases.

Under the circumstances, epimorphin involved in the control of the morphogenesis of epithelial tissue was isolated (EP0562123). This substance, being a physiologically active substance containing a protein consisting of 277 to 289 amino acids as a core protein, was revealed to be biosynthesized mainly by mesenchymal cells. It was also found that epimorphin had the action of promoting the morphogenesis of epithelial tissue through its action on epithelial cells, and that normal tissue formation was not progressed when epimorphin failed to function.

As to the structure of epimorphin, it has been found that epimorphin molecule can be roughly divided into four fragments from a structural viewpoint (European Patent Publication No. 0698666). That is, the polypeptide consisting of full length of epimorphin can be divided into the following four fragments, from its N-terminal, a coiled coil domain (1), a functional domain (2), a coiled coil domain (3), and hydrophobic domain at the C-terminal. In these fragments, it is suggested that the functional domain (the domain specified by 104th to 187th amino acids in human epimorphin) participates in cell adhesion and is closely related with an expression of physiological activity of epimorphin (the above-mentioned EP 0698666).

Since epimorphin has an action for promoting normal morphogenesis, this substance is expected to be useful as an active ingredient of medicaments for preventive or therapeutic treatment of diseases caused by abnormal morphogenesis, or medicaments such as a hair growth promoting agent.

However, a native epimorphin obtained from a mammal is almost insoluble in an aqueous media such as saline, which causes difficulty in practically using the substance as medicaments. Some attempts were made to produce epimorphin derivatives having good solubility while substantially keeping the promoting activity on morphogenesis of the native epimorphin. For example, International Publication WO01/94382 discloses pep7 as an example of such a peptide having a hair growth promoting activity. In such attempts to produce new epimorphin derivatives, it is essential to evaluate a hair growth promoting activity.

A specific method for evaluating a hair growth promoting activity is mentioned below. C3H and C57BL/6 mice are known to have sustained telogen for about 50 days from the 45th day after the birth to around the 95th day. Their hair cycle is easily judged based on the skin color changes, i.e., from pink in telogen to gray or black in anagen. A hair growth promoting activity can be evaluated by using this mice and evaluating whether or not the administration the test substance promotes the transition from telogen to anagen. However, it has been desired to develop a method for more simply and rapidly evaluating a hair growth promoting activity in vitro.

Disclosure of the Invention

An object of the present invention is to provide a method for the evaluation of hair growth promoting activity using an antibody which is useful for the evaluation of hair growth promoting activity and specifically recognizes a protein present in epithelial new follicle, and a kit for the evaluation of hair growth promoting activity comprising said antibody.

In order to achieve the above-mentioned object, the present inventors have carried out an intensive study, and have succeeded in identifying the amino acid sequence of a protein which is highly expressed at anagen stage of hair cycle and is useful for the judgment of hair growth inducing activity, and simply and rapidly evaluating the hair growth promoting activity by using an antibody against said protein, thereby completing the present invention.

Thus, in accordance with the present invention, there is provided a method for the evaluation of hair growth promoting activity wherein an antibody which recognizes a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, or a fragment thereof, is used. The aforementioned antibody includes an antibody which is obtained by immunizing an animal other than human with a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing.

Preferably, there is provided a method for the evaluation of hair growth promoting activity which comprises steps of incubating skin tissue pieces derived from living organism in the presence of a substance to be tested; recovering said skin tissue pieces, and reacting them with an antibody which recognizes a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, or a fragment thereof; and detecting or measuring said antibody or a fragment thereof which was reacted with the skin tissue pieces. The aforementioned antibody includes an antibody which is obtained by immunizing an animal other than human with a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing.

According to still another aspect of the present invention, there is provided a kit for the evaluation of hair growth promoting activity which comprises an antibody which recognizes a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, or a fragment

thereof. The aforementioned antibody includes an antibody which is obtained by immunizing an animal other than human with a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing.

Brief Description of the Drawings

Fig.1 shows the partial amino acid sequence of the antigen which is recognized by the antibody used in the present invention.

Fig. 2 shows the result of the evaluation of a hair growth promoting activity according to the method of the present invention.

Best Mode for Carrying out the Invention

The embodiments of the practice of the present invention are mentioned below in detail.

(1) A method for the evaluation of hair growth promoting activity wherein an antibody which recognizes a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, or a fragment thereof is used

The method for the evaluation of hair growth promoting activity according to the present invention is a method wherein an antibody which recognizes a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, or a fragment thereof, is used. More specifically, the hair growth promoting activity is evaluated by incubating skin tissue derived from living organism in the presence of a substance to be tested, recovering said skin tissue pieces, and reacting them with an antibody which recognizes a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, or a fragment thereof; and detecting or measuring said antibody or a fragment thereof which was reacted with the skin tissue pieces.

A protein of the amino acid sequence shown in SEQ ID NO: 1 of the sequence listing is registered in the database (NCIB (GeneBank)) under registration No. XM_177952 (*Mus musculus*, similar to trichohyalin). It has a small degree of homology with an inner root sheath protein "trichohyalin" of human hair follicles. The functions of this protein and the expression thereof have been totally unknown until now. It is, however, clarified by the present invention that the expression of this protein is highly specific for hair follicles in

anagen. As other proteins highly expressed in hair follicles in anagen, hair keratin and Hacl-1 have been known. A substance having hair growth inducing activity was added to a culture system of skin tissues, and an increase in the expression of these proteins was examined using an antibody. As a result, it was found that the expression of the protein of the present invention was most sensitive (increase in expression) to the addition of the hair growth-inducing substance in the above culture system.

The antibody used in the present invention is characterized in that it recognizes the protein of the amino acid sequence shown in SEQ ID NO: 1 of the sequence listing. The aforementioned antibody includes an antibody which is obtained by immunizing an animal other than human with a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing.

The antibody used in the present invention may be either a monoclonal antibody or a polyclonal antibody. The term "antibody" used in the present specification means not only a full-length antibody but also a fragment of the antibody. The fragment of the antibody is preferably a functional fragment, and its examples are $F(ab')_2$ and Fab' .

$F(ab')_2$ and Fab' are prepared by the treatment of immunoglobulin with protease such as pepsin or papain, and are antibody fragments produced by the digestion thereof at the sites which are before and after a disulfide bond existing between two H chains in a hinge region. The term "fragment of antibody" used in the present specification shall also include protein which contains an antigen-bonded site derived from a gene encoding said antibody.

For example, when IgG1 is treated with papain, cleavage takes place at the upper stream of the disulfide bond existing between the two H chains of the hinge region to give two homologous antibody fragments where L chain comprising VL (L chain variable region) and CL (L chain constant region) and H chain fragment comprising VH (H chain variable region) and CH γ 1 (γ 1 region in H chain constant region) are bonded by a disulfide bond at the C terminal region. Each of those two homologous antibody fragments is called Fab' . Further, when IgG is treated with pepsin, cleavage takes place at the downstream of the disulfide bond existing between the two H chains in the hinge region to give antibody fragments which are somewhat bigger than that where the above two Fab' are connected in a hinge

region. This antibody fragment is called F(ab')₂.

In the present invention, the aforementioned antibody may be used as an immobilized antibody immobilized on an insoluble carrier such as solid carrier, or may be used as a labeled antibody labeled with a labeling substance. An immobilized antibody is an antibody in a state of being carried on an insoluble carrier by physical adsorption, chemical bond or the like. Such an immobilized antibody may be used for detection, quantitative determination, separation or purification of antigen (i.e., a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing) contained in a sample (such as hair, follicles or an extract thereof). Examples of the insoluble carrier which can be used for immobilization of the antibody include (1) a container having an inner volume such as plate, test tube or tube, or beads, ball, filter, membrane and the like, each of which are made of water-insoluble substance such as plastics including polystyrene resin, polycarbonate resin, silicone resin or Nylon resin, or glass; (2) an insoluble carrier used for affinity chromatography, such as cellulose based carrier, agarose based carrier, polyacrylamide based carrier, dextran based carrier, polystyrene based carrier, polyvinyl alcohol based carrier, polyamino acid based carrier or porous silica based carrier.

A labeled antibody means an antibody labeled with a labeling substance, and such a labeled antibody may be used for detection or quantitative determination of antigen (i.e., a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing) contained in a sample (such as hair, follicles or an extract thereof). There is no particular limitation for the labeling substance used in the present invention, so far as its existence can be detected by bonding to an antibody by means of physical bonding, chemical bonding or the like. Examples of the labeling substance are enzyme, fluorescent substance, chemiluminescent substance, biotin, avidin or radioactive isotope. Specific examples include enzyme such as peroxidase, alkaline phosphatase, β -D-galactosidase, glucose oxidase, glucose-6-phosphate dehydrogenase, alcohol dehydrogenase, malic acid dehydrogenase, penicillinase, catalase, apoglucose oxidase, urease, luciferase or acetylcholine esterase; fluorescent substance such as fluorescein isothiocyanate, phycobilic protein, rare earth metal chelate, dansyl chloride or tetramethylol rhodamine isothiocyanate; radioisotope such as ³H, ¹⁴C, ¹²⁵I or ¹³¹I; biotin;

avidin; and chemiluminescent substance. With regard to a method for bonding a labeling substance to an antibody, known methods such as a glutaraldehyde method, a maleimide method, a pyridyl disulfide method and a periodic acid method may be used.

Here, each of the radioisotope and fluorescent substance is able to generate a detectable signal by itself, while each of enzyme, chemiluminescent substance, biotin and avidin is unable to generate a detectable signal by itself and, therefore, a detectable signal is generated as a result of reaction with one or more other substance(s). For example, in the case of an enzyme, at least a substrate is necessary and, depending upon a method for measuring the enzymatic activity (colorimetric method, fluorescent method, bioluminescent method or chemiluminescent method), various substrates are used. In the case of biotin, it is usual that at least avidin or enzyme-bound avidin is reacted therewith. If necessary, various coloring substances may be used depending upon the said substrate.

In the case where the antibody used in the present invention is a monoclonal antibody, the monoclonal antibody can be produced by using a hybridoma. The hybridoma can be produced by a conventional method as mentioned below.

First, mammal is immunized by using, as an immunogen, a protein having an amino acid sequence of SEQ ID NO.1 of the sequence listing or a sample containing said protein (for example, proteins extracted from hair collected from the skin of the growth period and/or follicles of whiskers of the growth period), whereby antibody-producing cells are prepared in the body of the animal. Although there is no particular limitation for the type of the mammal, the examples generally include mouse, rat, cattle, rabbit, goat and sheep, preferably rodents such as mouse, rat and rabbit, and more preferably, mouse or rat. Examples of the mouse are mouse of an A/J strain, a BALB/C strain, a DBA/2 strain, a C57BL/6 strain, a C3H/He strain, an SJL strain, an NZB strain or a CBA/JNCrj strain. Mouse of a BALB/C strain is preferred since the cell strain derived from myeloma of the same strain is established at the time of the preparation of hybridoma.

Before immunization, the immunogen may be mixed with an adjuvant for enhancing the immune response. Examples of the adjuvant include water-in-oil type emulsion (such as incomplete Freund adjuvant), water-in-oil-in-water type emulsion, oil-in-water type

emulsion, liposome, aluminum hydroxide gel, silica adjuvant, powdery bentonite and tapioca adjuvant, as well as cell body and cell wall of BCG, *Propionibacterium acnes*, etc. and somatic component such as trehalose dicholate (TDM); lipopolysaccharide (LPS) which is an endotoxin of Gram negative bacteria and lipid A fraction; β -glucan (polysaccharide); muramyl dipeptide (MDP); bestatin; synthetic compound such as levamisole; protein or peptidic substance derived from biocomponents such as thymus hormone, liquid factor of thymus hormone and taftsin; and a mixture thereof (such as complete Freund adjuvant). Such an adjuvant is effective for augmentation or suppression of immune response depending upon administration route, dose, administration period, and the like. In addition, depending upon the type of the adjuvant, difference is found in the production of antibody in blood to antigen, induction of cellular immunity, class of immunoglobulin, and the like. Therefore, it is preferred to suitably choose the adjuvant depending upon the aimed immune response. The method for the treatment with adjuvant is known in the art.

Immunization of mammal is carried out according to a method known in the art. For example, antigen is injected to mammal either subcutaneously, intracutaneously, intravenously or intraperitoneally. Since immune response varies depending upon the type and strain of the mammal to be immunized, an immunizing schedule is appropriately designed according to the animal to be used. Administration of antigen is repeatedly carried out for several times after the first immunization. Additional immunizations may be carried out, for example, after four weeks, six weeks and half a year from the first immunization.

After immunization, blood is collected from the mammal and the obtained blood is assayed for the presence of a hair follicle-binding activity to confirm the production of antibody against the follicles in the body of the mammal. The methods for the assay include known methods such as enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent antibody method

After confirming the production of follicle-binding antibody, a boost (additional injection of immunogen) can be carried out so that the immunocyte capable of producing a specific antibody is made into a state suitable for cell fusion. Although there is no particular limitation for the amount of the immunogen to be administered in the boost, it is preferred to

be about 4- to 5-fold of the initially immunized amount. Usually, a boost may be carried out using an emulsion of immunogen and incomplete Freund adjuvant. Route for the administration may be appropriately selected from subcutaneous, intracutaneous, intravenous, intraperitoneal administrations or the like.

After the final immunization, spleen cells are excised from the immunized mammal and subjected to a cell fusion with a cell strain derived from myeloma. In the cell fusion, it is preferred to use a cell strain having a high proliferation potency, and it is preferred that a cell strain derived from myeloma has a compatibility to the mammal from which the spleen cells to be fused is derived. Examples of the cell strain derived from myeloma of mouse include P3U1, P3X63-Ag8.653, Sp2/O-Ag14, FO.1, S194/5, XX0BU.1, P3/NS1/1-Ag4-1 and the like.

Cell fusion may be carried out by a known method in the art. Examples of the cell fusion method include a polyethylene glycol method, a method using Sendai virus, and a method using electric current.

The resulting fused cells may be proliferated by a condition known in the art. Desired fused cells are selected depending upon the binding ability of the produced antibody.

Binding ability of the antibody produced from the fused cells may be assayed according to a method known in the art. In the present invention, a cell strain of interest is cloned utilizing the selection depending upon the binding ability to follicles so as to obtain fused cells which produce an antibody which has a high binding ability and is specific to follicles. Binding ability of the antibody may be assayed by a method such as ELISA, RIA and fluorescent antibody method in the same way as in those mentioned already for the confirmation of production of antibody. Because of its simplicity and high sensitivity, ELISA is preferred.

Cloning of fused cells may be carried out by a method known in the art. The methods for cloning include a limiting dilution method, a soft agar method, and the like. Because of easily operation and high reproducibility, a limiting dilution method is preferred. In order to efficiently select useful cells from many fused cells obtained by cell fusion, it is preferred that selection of the cells is carried out from the initial stage of the cloning. In such a way, it

is possible to finally select a fused cell strain which produces an antibody having a desired binding ability.

By culturing the monoclonal antibody producing cell strain selected as mentioned above in a large scale, a monoclonal antibody used in the present invention can be produced in large amount. The methods for a large-scale culturing of the monoclonal antibody-producing cell strain include *in vivo* and *in vitro* culturing. An example of the large-scale *in vivo* culturing is a method where fused cells are intraperitoneally injected into mammal to proliferate so that an antibody is produced in abdominal dropsy. In the *in vitro* culturing, fused cells are cultured in a medium and an antibody is produced in the medium.

The monoclonal antibody of the present invention can be purified from the abdominal dropsy obtained by a large-scale culturing or from supernatant fluid of the culture medium by a known method in the art. For the purification, an appropriate combination of DEAE anion-exchange chromatography, affinity chromatography, ammonium sulfate fractionation, PEG fractionation, ethanol fractionation, and the like may be used. The antibody of the present invention may be purified preferably to a purity of about 90%, more preferably to a purity of about 95% or, still more preferably, to a purity of about 98%.

In the case of using a polyclonal antibody which recognizes a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, the polyclonal antibody can be produced by a conventional method.

For example, a mammal is immunized with a protein having an amino acid sequence of SEQ ID NO.1 of the sequence listing or a sample containing said protein (for example, proteins extracted from hair collected from the skin of the growth period and/or follicles of whiskers of the growth period) as an immunogen, and blood is collected from the mammal. Then, antibodies are separated and purified from the collected blood, and thereby a polyclonal antibody can be obtained. For example, mammals such as mouse, hamster, guinea pig, chicken, rat, rabbit, dog, goat, sheep and bovine can be immunized. The method of immunization is known to a skilled person in the art, and can be performed, for example, by administering an antigen once or more. For example, an antigen may be administered two or three times at an interval of 7 to 30 days. For example, a dose of about 0.05 to 2mg of

antigen can be used per administration. The route of administration is not specifically limited, and can be appropriately selected from subcutaneous, intracutaneous, intraperitoneal, intravenous, and intramuscular administrations. Administration by intravenous, intraperitoneal, or subcutaneous injection is preferred. Antigens may be used by dissolving in an appropriate buffer, for example an appropriate buffer containing a general adjuvant, such as complete Freund's adjuvant or aluminum hydroxide. There may be cases where the above adjuvant may not be used depending on administration routes, conditions or the like.

An immunized mammal is reared for certain period, a serum of the mammal is sampled, and then the antibody titer is measured. When the antibody titer starts to elevate, booster immunization is performed by using, for example, 10 μ g to 1000 μ g of the antigen. Blood is collected from an immunized mammal 1 to 2 months after the final administration. The collected blood is separated and purified by standard techniques including centrifugation, precipitation using ammonium sulfate or polyethylene glycol, and chromatography such as gel filtration chromatography, ion exchange chromatography and affinity chromatography. As a result, polyclonal antibodies which recognize the protein of the present invention can be obtained as a polyclonal anti-serum. The complement system can be inactivated by treating the anti-serum, for example, at 56°C for 30 minutes.

The method for the evaluation of hair growth promoting activity according to the present invention may be any method so far as it is an assay using the aforementioned antibody or, in other words, an immunoassay. Examples thereof include western blotting, enzyme-linked immunosorbent assay (ELISA), fluoroimmunoassay, radioimmunoassay (RIA), luminoimmunoassay, immunoenzymatic assay, immunofluorescence assay, immunoturbidimetry, latex agglutination reaction, latex turbidimetry, erythrocyte agglutination reaction and particle agglutination reaction.

There is no particular limitation for the type of the test substance which is subjected to the method for the evaluation of the present invention, and the test substance may be either oligopeptides or low-molecular organic compounds. For example, there may be used an oligopeptide having a partial amino acid sequence of epimorphin which has been known to have a hair growth promoting activity.

When the method for the evaluation of hair growth promoting activity according to the present invention is carried out by means of an immunoassay using labeled antibody such as enzyme-linked immunosorbent assay, fluoroimmunoassay, radioimmunoassay or luminoimmunoassay, it is also possible to carry out the assay by a sandwich method or a competition method. In the case of a sandwich method, at least one of solid phase antibody and labeled antibody is the antibody of the present invention.

With regard to the solid phase carrier, there may be used the above-mentioned carriers which are described in the present specification as specific examples for the insoluble carrier in relation to the immobilized antibody. Also with regard to the labeled substance, there may be used the above-mentioned substances which are described in the present specification in relation to the labeled antibody.

A method for the measurement may be carried out by a known method ("Enzyme-Linked Immunosorbent Assay", Supplementary Issue No. 31 of *Tampakushitsu, Kakusan, Koso*, edited by Tsunehiro Kitagawa, et al., published by Kyoritsu Shuppan, 1987).

For example, a solid phase antibody is reacted with a sample and a labeled antibody at the same time, or a solid phase antibody is reacted with a sample, and after being washed, it is reacted with a labeled antibody, thereby forming a complex of solid phase antibody-antigen-labeled antibody. Then, unbound labeled antibody is separated by washing, and the amount of the antigen in the sample can be measured from the amount of the bound labeled antibody. Specifically, in the case of the enzyme-linked immunosorbent assay (ELISA), the labeled enzyme is reacted with a substrate under an optimum condition and the amount of the reaction product is measured by, for example, an optical method. In the case of the fluoroimmunoassay, intensity of fluorescence by a fluorescent substance labeling is measured, and in the case of the radioimmunoassay, radiation dose by a radioactive substance labeling is measured. In the case of the luminoimmunoassay, amount of luminescence in the luminous reaction system is measured.

In the method for the detection and/or the quantitative determination according to the present invention, when the production of an immune complex aggregate by immunoturbidimetry, latex agglutination reaction, latex turbidimetry, erythrocyte

agglutination reaction, particle agglutination reaction or the like is measured by measuring its transmitting light or scattering light by an optical method, or is measured visually, it is possible to use phosphate buffer, glycine buffer, Tris buffer, Good buffer or the like as a solvent, and a reaction promoting agent such as polyethylene glycol or a non-specific reaction suppressing agent may be contained therein.

When an antibody is used by sensitizing it to a solid phase carrier, there may be used particles made of the material such as polystyrene, styrene-butadiene copolymer, (meth)acrylate polymer, latex, gelatin, liposome, microcapsule, erythrocyte, silica, alumina, carbon black, metal compound, metal, ceramics or magnetic substance as a solid phase carrier.

The methods for the sensitization include known method such as physical adsorption, chemical bonding or a combination thereof. A method for the measurement may be carried out by a known method. For example, in the case of the measurement by an optical method, the sample is reacted with the antibody, or the sample is reacted with the antibody which was sensitized with a solid phase carrier. Then, the transmitting light or the scattering light is measured by an end-point method or a rate method.

When the measurement is carried out visually, the sample is reacted with the antibody sensitized with a solid phase carrier in a container such as a plate or a microtiter plate, and the state of agglutination is judged visually. Instead of measuring visually, the measurement may be carried out by an instrument such as a microplate reader.

(2) Kit for evaluation of hair growth promoting activity comprising an antibody which recognizes a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, or a fragment thereof

The kit of the present invention comprises an antibody which recognizes a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, or a fragment thereof, which is provided by the present invention. The monoclonal antibody, polyclonal antibody or fragment thereof mentioned herein may be the immobilized antibody or the labeled antibody which was mentioned hereinabove.

For example, when an antibody which recognizes a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing which is provided by the present invention is used as a primary antibody, the kit of the present invention may further comprise a secondary antibody for the detection of a complex formed by the antigen-antibody reaction. The kit of the present invention may still further comprise various auxiliary agents in addition to those antibodies, so that the said kit can be utilized efficiently and easily. Examples of the auxiliary agent include those which are commonly used in a kit of reagents for immunological measurement, such as a solubilizer for dissolving the solid secondary antibody, a detergent used for washing the insoluble carrier, a substrate for measuring the enzymatic activity when enzyme is used as a labeling substance for the antibody, and a reaction stopping agent. The kit of the present invention may also comprise the instructions for carrying out the evaluation of hair growth promoting activity.

Examples

The present invention will be explained more specifically by the following Examples. However, the scope of the invention is not limited to these examples.

Example 1: Protein expressed at a high level in anagen stage of hair cycle

(1) Preparation of expression library

Using TRIZOL (GIBCO 15596-018) in accordance with a manual attached therewith, total RNA was prepared from the back of a C57BL mouse, the hair cycle of which was in anagen (35 days after birth). Using Quick PrepTM micro mRNA purification kit manufactured by Amersham Pharmacia, mRNA (20 µg) was prepared from the total RNA. Thereafter, cDNA was synthesized from 5 µg of the mRNA with random primers, employing Time Saver cDNA Synthesis kit 27-9262-01 manufactured by Amersham Pharmacia. Due to the use of adaptors attached with the aforementioned kit, the synthesized cDNA had *Eco*RI and *Not*I sites at both termini thereof. The obtained cDNA was inserted into λExCell *Eco*RI/CIP (27-5011-01) manufactured by Amersham Pharmacia, so as to produce an expression library.

Escherichia coli MN522 was infected with the thus produced expression library, and the obtained plaques were dispersed on an LB plate at a concentration of 20,000 plaques/plate. The LB plate was then immersed in a 10 mM IPTG solution, followed by air drying. Thereafter, the LB plate was covered with nitrocellulose, and it was left at rest overnight.

(2) Preparation of a monoclonal antibody which is specific for new follicle

Hairs were cut off from the skin of B57BL mouse of growing stage (48 to 50 days), and were incubated overnight at 37°C in PBS containing 8M urea, 2% SDS and 100mM DTT, thereby the protein was extracted. Further, whisker follicles of B57BL mouse (where hair ball portion is stained with pigment; growing stage (anagen)) were collected with a stereoscopic microscope, and were homogenized in PBS. The above 2 samples (0.5mg of protein weight) were mixed, and mixed with the same amount of the complete adjuvant to prepare micelle.

The above-obtained micelle (0.2mg) was subcutaneously (3 sites) administered to a rat (Wister) for immunization. After the first immunization, the booster was performed in the same way as in the above. After 2 weeks, the second booster was performed in the same way as in the above. On the third day after the second booster, a spleen was removed from the immunized rat, and the blood cells were collected by mesh. Antibody producing cells are contained in these blood cells. All amount of the above-collected blood cells were mixed with mouse myeloma P3U1 using polyethyleneglyco 1500, and were suspended in Dulbecco/Hum F12 mixed medium (10^7 cells/ml). 100 μ l of the culture were inoculated in each well of 96-well plate. On the next day, the same amount (100 μ l) of HAT medium (Sigma) was added to each well. After 2 days, 150 μ l of the medium was removed under aspiration from each well, and 150 μ l of the fresh medium was added to each well. The 96-well plate was placed in CO₂ incubator at 37°C.

The follicles of the growing whisker of B57BL mouse were dissolved in 8M urea by ultrasonic treatment. A nitrocellulose membrane was immersed in this solution for 5 minutes, and was washed well with PBS. Biorad dot blotter equipped with the above membrane was used to perform the first screening of the hybridoma supernatant which was recovered from

each well of the above 96-well plate. First, the above prepared nitrocellulose membrane which was equipped in Biorad dot blotter was blocked with Tris buffer containing 5% skim milk (TBST), and then 100 μ l of the hybridoma supernatant was added to each well. After incubation for 1 hour, the wells were washed with Tris buffer, and the second antibody, horseradish peroxidase labeled anti-rat IgG (1 μ g/ml in TBST), was added. ECL agent (Amersham Pharmacia), which is a coloring substrate, was added, and 50 antibodies in total which reacted with the growing whisker follicle were selected by detection of coloring (first screening).

Among the 50 antibodies selected in the above first screening, the antibodies which specifically reacted with the frozen segment (10 μ m) of the growing whisker follicle were selected (second screening). Specifically, the frozen segment of the growing whisker follicle was placed on a slide glass, and the hybridoma supernatant selected in the first screening was added thereto, and the coloring was developed with the second antibody. More specifically, the frozen segment of the growing whisker follicle which was prepared by Cryosdat (Bright) was treated with methanol at -20°C, and was blocked with TBST for 1 hour, and then was reacted with the hybridoma supernatant for 1 hour. The sample was washed with Tris buffer, and was reacted with FITC-labeled anti-rat IgG (100 μ g/ml in TBST). The sample was washed with Tris buffer, and was covered with a cover glass. The observation was carried out under fluorescent microscope.

As a result of the second screening, 8 antibodies were selected. These antibodies do not react with epidermis, and specifically react with follicle. These 8 antibodies were cloned by the limiting dilution.

The reactivity of these 8 antibodies was examined by Western Blotting using growing whisker follicle or resting whisker follicle as a sample, and using slide samples of the skins having follicles derived from 14 day fetal mouse. As a result, mAb27 was obtained as a monoclonal antibody which specifically reacted with growing whisker follicle and plastic follicle (new follicle), and did not react with resting whisker follicle. The hybridoma which produced monoclonal antibody mAb27 was deposited with Patent and Bio-Resource Center of National Institute of Advanced Industrial Science and Technology (Chuo-6, 1-1, Higashi

1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on November 2, 2001 under the deposit number of FERM P-18578, and this deposit was transferred to the International deposit on July 22, 2002 under the deposit number of FERM BP-8121.

(3) Immunoscreening

The *Escherichia coli* expression library produced in (1) above was subjected to immunoscreening using the monoclonal antibody mAb27 produced in (2) above. Specifically, a library of 300,000 clones was developed (20,000 plaques/plate), and nitrocellulose into which IPTG had been infiltrated was then made to closely come into contact with the plaques, so that expressed gene products were transcribed onto the nitrocellulose. The nitrocellulose was blocked with 1% skim milk/TBS (STBS). Thereafter, it was incubated with 10 µg/ml mAb27 and HRP-labeled anti-rat IgG (Amersham), followed by detection with ECL (Amersham).

The thus obtained 6 positive clones were recovered, and their nucleotide sequences were determined. As a result, it was found that all 6 clones shared a common sequence (Figure 1).

Example 2: Database search

The database (NCIB (GeneBank)) was searched for the common sequence (Figure 1) identified in Example 1. As a result, a registration No. XM_177952 (*Mus musculus*, similar to Trichohyalin) was found. The amino acid sequence of registration No. XM_177952 is shown in SEQ ID NO: 1, and the nucleotide sequence thereof is shown in SEQ ID NO: 2. The amino acid sequence shown in Figure 1 corresponds to a portion at positions 800 to 1135 of the amino acid sequence shown in SEQ ID NO: 1. However, Leu at position 1133 of the amino acid sequence shown in SEQ ID NO: 1 is substituted by Gln in Figure 1, and Arg at position 1135 of the amino acid sequence shown in SEQ ID NO: 1 is substituted by His in Figure 1.

Example 3: Production of a recombinant protein

(1) Preparation of full-length cDNA by PCR

A cDNA library was produced from the mRNA of the skin in the growth period, using oligo dT primers and reverse transcriptase. Using the obtained cDNA library as a template, PCR was carried out with two types of primers (5'-atgtctccactataagaagcattgtagat-3' (SEQ ID NO: 3) and 5'-ttaagggcgggtattgagacctctgctcctg-3' (SEQ ID NO: 4)), so that full-length cDNA was cloned. LA TaqTM kit (Takara) was used in the PCR reaction, and the PCR conditions consisted of: 94°C, 1 minute; 94°C, 30 seconds; 60°C, 30 seconds; and 72°C, 2 minutes, so that the cDNA of interest was amplified by 30 cycles.

The obtained clone contained full-length cDNA encoding the amino acid sequence shown in SEQ ID NO: 1.

(2) Transformation

The PCR product obtained in (1) above was inserted into a pTarget vector (Promega), so as to construct a recombinant expression vector. Thereafter, COS-1 cells as host cells were transfected with the recombinant expression vector. Transfection was carried out using Lipofectamine (GIBCO) in accordance with the manual attached therewith. The COS-1 cells were cultured in a Dalbecco's Ham's F12 medium to which 10% FCS had been added.

(3) Extraction of recombinant protein

(a) Preparation of affinity column

Afigel 10 (Biorad) was adjusted to 4°C, so as to make the gel homogenous. The gel was then placed in a Buchner's funnel, followed by filtration. Thereafter, the gel was washed with 3 times its volume of deionized water at 4°C. After washing, the gel was placed in a flask, and an antibody solution obtained by dissolving a monoclonal antibody mAb27 in PBS (wherein 0.5 ml of an antibody solution was used per 1 ml of the gel) was added into the flask. The mixture was fully stirred and suspended. While slowly stirring the suspension with a stirrer, it was reacted at room temperature for 1 hour. The supernatant was eliminated by centrifugation, and the reaction solution was then adjusted with PBS to 10 ml. 0.1 ml of 1M glycine ethyl ester (pH 8) per ml of the gel was added, and the mixture was allowed to react for 1 hour. After completion of the reaction, the gel was packed into an Ecopac column (available up to 20 ml, Biorad), and it was then washed with distilled water until no reaction products were detected by OD₂₆₀. Finally, it was washed with an eluent (10 mM Tris-HCl, 1

mM MgCl₂, 1 mM EDTA, protein inhibitor cocktail (product name: CompleteMini; Roche), 0.15 M NaCl, and 0.5% TritonX-100; adjusted to pH 3.0 with HCl).

(b) Purification of antigen

The transformed COS-1 cells produced in Example 2, in which full-length cDNA was expressed, were recovered on the third day after the transfection, and washed with PBS. Thereafter, the cells were added to 100 ml of a mixed solution of 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EDTA, and protein inhibitor cocktail (product name: CompleteMini; Roche). Then, they were fully crushed with a homogenizer. Subsequently, NaCl was added thereto to a final concentration of 0.15 M, and TritonX-100 was then added thereto to a final concentration of 0.5%. The obtained mixture was stirred at 4°C for 3 hours with a stirrer. The mixture was then centrifuged at 25,000 rpm for 10 minutes, and the supernatant was recovered. Thereafter, a protein was extracted from the cells. 100 ml of the obtained extract was subjected to the affinity column of (1) above.

Washing was carried out with a washing solution (10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EDTA, protein inhibitor cocktail (product name: CompleteMini; Roche), 0.15 M NaCl, and 0.5% TritonX-100). Thereafter, an eluent (a solution obtained by adjusting the above washing solution to pH 3.0 with HCl) was added to the column. The eluant was recovered in amounts of 2 ml each. A protein-containing fraction was then recovered by absorption at OD 280 nm.

(c) Measurement of molecular weight of purified antigen by electrophoresis

The protein-containing fraction recovered in (b) above was electrophoresed by SDS-PAGE, and then, Western blotting was carried out using a monoclonal antibody mAb27. As a result, a band was detected at the position of 220 kDa. In addition, the gel which was obtained by the electrophoresis of the protein-containing fraction recovered in (b) above by SDS-PAGE, was stained with Coomassie blue, and the band of 220 kDa was cut out, so as to prepare a purified antigen.

Example 4: Production of polyclonal antibody which recognizes a protein of amino acid sequence shown in SEQ ID NO: 1 of the sequence listing

The purified antigen prepared in Example 3 (that is, the excised gel containing the protein of the amino acid sequence shown in SEQ ID NO: 1) was recovered and placed in 20% ethanol, and then left overnight. Thereafter, the gel was recovered. A sample containing approximately 100 μ g or less of the protein was mixed with Titer Max Gold (an adjuvant from CytRX Corp.) to produce an emulsion. A rat was immunized with the emulsion for 2 months (3 times in total). Two months after the primary immunization, the blood was collected from the rat, and the serum was then prepared by common methods, so as to obtain a polyclonal antibody which recognizes the protein of the amino acid sequence shown in SEQ ID NO: 1.

Example 5: Evaluation of hair growth-promoting activity, using polyclonal antibody which recognizes a protein of amino acid sequence shown in SEQ ID NO: 1 of the sequence listing

Differentiation of human keratinocytes was evaluated. Human keratinocytes (which are skin cells containing epidermal keratinocytes and hair follicle-derived cells) were purchased from Sanko Junyaku. A peptide ssb7 (which was obtained by biotinating the N-terminus of Ser-Ile-Glu-Gln-Ser-Cys-Asp-Gln-Asp-Glu (SEQ ID NO: 5) (NHS-Biotin, Pierce) and then subjecting it to S-S crosslinking) was added to a culture solution attached with the purchased cells, such that the concentration became 20 μ M. It was cultured on a 96-well plate for 1 week. Thereafter, cells were recovered from the wells. The cells were placed in 100 μ l of an SDS sample buffer (0.02 g/ml SDS, 0.2 g/ml glycerol, pH 6.8), and they were then dissolved in the buffer using an ultrasonic disintegrator. A control (human keratinocytes that were cultured for 1 week without adding ssb7) was treated in the same manner as described above. A solution obtained by the above treatment was electrophoresed (35 mA, 1.5 hours) by SDS-PAGE (4% to 20% acrylamide), and the resultant product was transferred to a PVDF membrane, followed by incubation in a Tris buffer solution (TBST) containing 5% skim milk for 1 hour. It was allowed to react for 1 hour with a polyclonal antibody (obtained by diluting serum to 1/100) against a protein having an amino acid sequence shown in Figure 1, which was a partial sequence of the amino acid sequence shown in SEQ ID NO: 1. Thereafter, the reaction product was fully washed with TBS. It was then

allowed to react with a secondary antibody obtained by diluting peroxidase-labeled anti-rat IgG (Amersham) with TBST to 1/1,000. Thereafter, the resultant product was fully washed, and the level of the reactivity of the polyclonal antibody was examined using an ECL kit (Amersham).

The obtained results are shown in the right figure of Figure 2.

In the right figure of Figure 2, the right lane represents the results of the control, and the left lane represents the results obtained by addition of EPM (ssb7), which was a hair growth tonic. AHF in the figure represents a protein of the amino acid sequence shown in SEQ ID NO: 1 of the sequence listing. As is clear from the results in Figure 2, in the sample to which EPM (ssb7) as a hair growth tonic had been added, a band was detected, which was expressed more highly than that of the control. This result reflects that the expression level of the protein of the amino acid sequence shown in SEQ ID NO: 1 of the sequence listing was increased.

The expression of the protein of the amino acid sequence shown in SEQ ID NO: 1 was analyzed at the mRNA level by Northern blotting in the same manner as described above. Specifically, first, 10 µg of total RNA prepared from the back skin of a rat was electrophoresed in agarose gel, and the resultant sample was transferred to a nylon membrane (HibondN+, Amersham). Subsequently, a label was introduced into the translation region of AHF cDNA using DIG (manufactured by Roche). Using this as a probe, AHF mRNA that had been transferred to the aforementioned nylon membrane was detected.

The obtained results are shown in the left figure of Figure 2. As in the case of the right figure of Figure 2, in the sample to which EPM (ssb7) as a hair growth tonic had been added, a band that was stronger than that of the control was detected.

Industrial Applicability

According to the method of the present invention, the hair growth promoting activity of a test substance can be efficiently evaluated.

CLAIMS

1. A method for the evaluation of hair growth promoting activity wherein an antibody which recognizes a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, or a fragment thereof, is used.

2. The method for the evaluation of hair growth promoting activity according to claim 1 wherein an antibody which is obtained by immunizing an animal other than human with a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, or a fragment thereof, is used.

3. The method for the evaluation of hair growth promoting activity according to claim 1 which comprises steps of;

incubating skin tissue pieces derived from living organism in the presence of a substance to be tested;

recovering said skin tissue pieces, and reacting them with an antibody which recognizes a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, or a fragment thereof; and

detecting or measuring said antibody or a fragment thereof which was reacted with the skin tissue pieces.

4. The method for the evaluation of hair growth promoting activity according to claim 3 wherein an antibody which is obtained by immunizing an animal other than human with a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, or a fragment thereof, is used.

5. A kit for the evaluation of hair growth promoting activity which comprises an antibody which recognizes a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, or a fragment thereof.

6. The kit for the evaluation of hair growth promoting activity according to claim 5 which comprises an antibody which is obtained by immunizing an animal other than human with a protein of amino acid sequence of SEQ ID NO.1 of the sequence listing, or a fragment thereof.