

Description

Title of Invention: METHOD FOR SCREENING COMPONENT THAT AFFECTS FLUCTUATION OF ADHESION PROTEINS IN STRATUM CORNEUM CELLS

Technical Field

[0001] The present invention relates to a method for screening an component that affects the increase or decrease of adhesive protein in stratum corneum cells, and a method for preparing a stratum corneum cell sample suitable for the screening method.

Background Art

[0002] An adhesion device for keratinocytes is typically desmosome. The desmosome is a structure participating in adhesion between epidermal cells and between stratum corneum cells, and it is known that in the adhesion of epidermal cells through desmosome, the desmosome cooperates with a cytoskeleton composed of keratin filaments, and strong construction in the epithelial tissue is maintained.

[0003] Regarding deterioration of skin condition such as desquamation of stratum corneum due to skin roughening, sunburning or drying, an increase in desmosome protein is known as one of the causes.

As a method for controlling the amount of desmosome protein, a method in which desmosome protein accumulated in the stratum corneum is decomposed by protease to improve acne, dandruff or desquamation has been reported so far (Patent Literature 1).

[0004]As constituent proteins of desmosome, desmoglein 1, 2, 3, 4, desmocollin 1, 2, 3, desmoplakin 1, 2, etc. are known.

Particularly in the desmoglein 1 of adhesive protein among the constituent proteins of desmosome, there is a close correlation between protein decomposition of the desmosome component and cell exfoliation. On this account, to confirm the existence state of desmoglein in the stratum corneum is to evaluate the desmosome function, so that this is extremely important in order to evaluate the skin condition.

Citation List

Patent Literature

[0005] Patent Literature 1

Japanese Translation of PCT International
Application Publication No. 1995-505383

Summary of Invention

Technical Problem

[0006] From the above, search for an ingredient that decreases the amount of desmosome or adhesive protein for constituting desmosome has been desired.

As a method for screening such an ingredient, there is a method including collecting stratum corneum cells using a tape stripping method, transferring the cells onto a glass slide to prepare a stratum corneum cell sample, staining adhesive protein by an immunohistochemical staining method and observing the stained adhesive protein by an arbitrary microscope.

[0007] By the way, in the transfer of the stratum corneum cells using the tape stripping method, an ingredient of the adhesive tape remains on the glass slide, and if this is stained, there is a fear that the adhesive protein cannot be discriminated from this, so that the glass slide needs to be cleaned.

However, if the glass slide is cleaned, the stratum corneum cells having been transferred are also detached, and evaluation using, as an index, the abundance of the adhesive protein present in the stratum corneum cells is difficult, so that it cannot be said that this method is a satisfactory screening method.

[0008] An object of the present invention is to provide a method for screening an ingredient that affects the increase or decrease of adhesive protein present in stratum corneum cells, such as desmoglein, the screening method being more accurate than conventional methods.

Another object of the present invention is to provide a method for preparing a stratum corneum cell sample for evaluating the abundance of the adhesive protein more accurately in the aforesaid screening method.

Solution to Problem

[0009] The present invention to solve the above problem is a method for screening an ingredient that affects the increase or decrease of adhesive protein in stratum corneum cells, comprising:

- a collecting step of collecting stratum corneum cells using a tape stripping method,

- a transfer step of transferring stratum corneum onto a glass slide having a positively charged surface,

- a cleaning step of cleaning the glass slide,

- a preparation step of adding a search target ingredient to the glass slide and staining adhesive protein by an immunohistochemical staining method to prepare a screening sample,

- an image acquiring step of photographing an arbitrary place of the screening sample to acquire an image for analysis, and

- an evaluation step of evaluating an action of the search target ingredient to inhibit formation of compact stratum corneum using, as an index, an area of a stained part in the image for analysis.

[0010] It is known that the cell surface has a negative charge (COO^-) due to a carboxyl group of a phospholipid.

According to the screening method of the present invention, by transferring stratum corneum cells onto a glass slide having a positively charged surface, detachment of the stratum corneum cells due to cleaning can be prevented, and the abundance of adhesive protein in the stratum corneum cells can be more accurately evaluated.

[0011] In a preferred embodiment of the present invention, in the preparation step, a screening control sample to which the search target ingredient has not been added is prepared, and

the evaluation step is a step of evaluating the search target ingredient as having an action to decrease the amount of adhesive protein when an area of a stained part of the screening sample and an area of a stained part of the screening control sample are compared with each other to find that the area of the stained part of the screening sample is smaller.

By adopting such an embodiment, an ingredient that affects the increase or decrease of the adhesive protein can be more accurately screened.

[0012] In a preferred embodiment of the present invention, in the preparation step, a plurality of screening samples identical in type of the search target ingredient and different in concentration thereof is prepared, and

the evaluation step is a step of evaluating an optimum concentration in the action to decrease the amount of adhesive protein by comparing areas of stained parts of the plurality of the screening samples.

By adopting such an embodiment, not only screening of an ingredient having an action to decrease the amount of adhesive protein but also evaluation of its optimum concentration can be carried out.

[0013] In a preferred embodiment of the present invention, the adhesive protein is desmoglein 1.

[0014] In a preferred embodiment of the present invention, the glass slide is a glass slide having NH_3^+ arranged on a surface thereof.

Since NH_3^+ forms an ionic bond together with COO^- on the cell surface, it can improve adhesion property of the stratum corneum cells to the glass slide.

[0015] In a preferred embodiment of the present invention, the cleaning step is a step of immersing the glass slide in xylene.

By cleaning the glass slide through immersion of the glass slide in xylene, detachment of stratum corneum cells can be further inhibited.

[0016] The present invention to solve the above problem is a method for preparing a stratum corneum cell sample using a tape stripping method, comprising:

a transfer step of transferring stratum corneum onto a glass slide having a positively charged surface, and

a cleaning step of cleaning the glass slide.

By transferring stratum corneum onto the glass slide having a positively charged surface, detachment of the stratum corneum cells from the glass slide in the subsequent cleaning step can be inhibited.

[0017] In a preferred embodiment of the present invention, the glass slide is a glass slide having NH_3^+ arranged on a surface thereof.

Advantageous Effects of Invention

[0018] According to the screening method of the present invention, an ingredient that affects the increase or decrease of adhesive protein can be more accurately screened than in conventional methods.

According to the method for preparing stratum corneum cells of the present invention, detachment of stratum corneum cells from the glass slide can be inhibited.

Brief Description of Drawings

[0019] [Figure 1] Figure 1 is a flowchart illustrating a screening method of the present embodiment.

[Figure 2] Figure 2 is a flowchart illustrating a stratum corneum cell sample preparation step in the present embodiment.

[Figure 3] Figure 3 is a flowchart illustrating an evaluation step in the present embodiment.

[Figure 4] Figure 4 shows microscope images of screening samples in which a wild thyme extract or a Rosa roxburghii extract has been added to stratum corneum cell samples as a search target ingredient, and a screening control sample.

[Figure 5] Figure 5 is a graph showing ratios of areas of stained parts in the screening samples when assuming that the area of a stained part in the screening control sample is 100%.

[Figure 6] Figure 6 is a group of drawings (typical) showing measurement results of the amount of desmoglein 1 in Example 2.

[Figure 7] Figure 7 is a graph showing measurement results of the amount of desmoglein 1 in Example 2.

[Figure 8] Figure 8 is a graph showing measurement results of L* value in Example 3.

[Figure 9] Figure 9 is a graph showing measurement results of a* value in Example 3.

[Figure 10] Figure 10 is a graph showing measurement results of b* value in Example 3.

[Figure 11] Figure 11 is a graph showing measurement results of hardness in Example 3.

[Figure 12] Figure 12 is a graph showing measurement results of the amount of desmoglein 1 in Example 3.

[Figure 13] Figure 13 is a group of drawings (typical) showing measurement results of the amount of desmoglein 1 in Example 3.

[Figure 14] Figure 14 is a group of drawings (typical) showing measurement results of the amount of desmoglein 1 in Example 4.

[Figure 15] Figure 15 is a graph showing measurement results of the amount of desmoglein 1 in Example 4.

[Figure 16] Figure 16 shows optical microscope images obtained by observing the state of exfoliation of stratum corneum cells in Example 4.

[Figure 17] Figure 17 is a group of drawings (typical) showing measurement results of the amount of desmoglein 1 in Example 5.

[Figure 18] Figure 18 is a graph showing measurement results of the amount of desmoglein 1 (6 weeks) in Example 5.

[Figure 19] Figure 19 is a graph showing measurement results of the amount of desmoglein 1 (12 weeks) in Example 5.

[0020] Hereinafter, the screening method of the present invention will be described in detail with reference to the flowcharts of Figures 1 to 3.

[0021] The screening method of the present invention includes a stratum corneum cell sample preparation step S10, a screening sample preparation step S20, an image acquiring step S30, and an evaluation step S40.

[0022] (1) Stratum corneum cell sample preparation step

In Figure 2, a flowchart illustrating the step S10 in detail is shown.

In the step S10, a collecting step S11 of collecting stratum corneum cells by a tape stripping method is carried out.

The tape stripping method is a generic term for methods for exfoliating cells of the skin by applying a pressure-sensitive adhesive, typically a pressure-sensitive adhesive tape, to the skin and stripping it.

The pressure-sensitive adhesive used is not specifically limited, but from the viewpoint of convenience, a cellophane tape is preferably used.

[0023] Subsequently, a transfer step S12 of transferring the stratum corneum cells having been collected in the step S11 onto a glass slide having a positively charged surface is carried out.

[0024] The surface of a human cell such as a stratum corneum cell is negatively charged by the influence of a carboxyl group (COO^-) of a phospholipid that forms a lipid bilayer membrane.

By transferring the stratum corneum cells onto the glass slide having a positively charged surface in the step S12, the stratum corneum cells are rarely detached from the glass slide owing to the electrostatic interaction.

[0025] Examples of the glass slides having a positively charged surface include glass slides whose surfaces have been modified with polylysine, polyalkylamine or amino-modified silicone.

In the present invention, it is preferable to use a glass slide having NH_3^+ arranged on its surface.

Since NH_3^+ forms an ionic bond together with a carboxyl group of a phospholipid, the stratum corneum cells are more rarely detached from the glass slide.

[0026] As the glass slide having NH_3^+ arranged on its surface, a commercial product can be used, and examples thereof include "PLL Coated Glass Slide" (manufactured by Matsunami Glass Ind., Ltd.), "APS Coated Glass Slide" (manufactured by Matsunami Glass Ind., Ltd.), and "MAS coated Glass Slide" (manufactured by Matsunami Glass Ind., Ltd.).

[0027] Subsequently, a cleaning step S13 of cleaning the glass slide onto which the stratum corneum cells have been transferred is carried out.

A method for cleaning the glass slide is not particularly limited, and the glass slide can be cleaned using a cleaning machine of shaking type or ultrasonic type.

In the present invention, it is preferable to clean the glass slide by immersing it in a cleaning agent containing an organic compound.

By adopting such an embodiment, detachment of the stratum corneum cells can be further inhibited.

[0028] Examples of the cleaning agents include organic solvents such as xylene, chloroform and ethyl acetate,

but it is preferable to use xylene not affecting the amount of adhesive protein of the stratum corneum cells.

[0029] When cleaning by immersion is carried out, the immersion time at room temperature is preferably 3 hours or more, more preferably 6 hours or more, still more preferably 12 hours or more, and particularly preferably 24 hours or more.

[0030] A stratum corneum cell sample is prepared through the aforesaid steps, and subsequently, the screening sample preparation step S20 is carried out.

In the step S20, an ingredient that becomes a search target (referred to as a "search target ingredient" hereinafter) is added to the stratum corneum cell sample.

[0031] The search target ingredient is not particularly limited, but in the light of application of an ingredient having an action to decrease the amount of adhesive protein to humans, for example, ingredients applicable to cosmetics and ingredients applicable to foods can be used as the search target ingredients.

Examples of such ingredients include plant extracts.

[0032] The search target ingredient is preferably diluted with phosphate-buffered saline (PBS) according to the concentration at which the screening is carried out.

[0033] In one embodiment of the present invention, a screening control sample containing no search target ingredient may be prepared.

Such an embodiment is suitable for screening the type of an ingredient having an action to decrease the amount of adhesive protein.

[0034] In one embodiment of the present invention, a plurality of screening samples which is identical in type of the search target ingredient and different in concentration thereof may be prepared.

Such an embodiment is suitable for screening an optimum concentration at which an action to decrease the amount of adhesive protein is exhibited.

[0035] In the step S20, the stratum corneum cells to which the search target ingredient has been added is stained by an immunohistochemical staining method.

Staining by the immunohistochemical staining method can be carried out in a conventional way, and any one of a direct method, an indirect method and a sensitization method can be arbitrarily selected according to the type of adhesive protein.

Further, an arbitrary blocking agent can be used as needed.

[0036] The adhesive protein that becomes a staining object is not particularly limited as long as it is contained in the stratum corneum cells, but it is preferably desmoglein 1.

Regarding the desmoglein 1, there is a close correlation between protein decomposition of the desmosome component and cell exfoliation, and screening

of an ingredient that affects the increase or decrease of the amount thereof is extremely useful.

[0037] Subsequently, the image acquiring step S30 of photographing an image of the stratum corneum cells in which the adhesive protein has been stained is carried out.

The photographing of an image is carried out using a microscope. The type of the microscope is not particularly limited, and an appropriate microscope can be used according to the immunohistochemical staining method that has been carried out in the step S20.

Examples of the microscopes include an optical microscope, a confocal laser scanning microscope, an optical microscope, and an electronic microscope.

[0038] In the present invention, it is preferable to use the confocal laser scanning microscope.

By using the confocal laser scanning microscope, a boundary between the stained part (adhesive protein) and the unstained part (part other than adhesive protein, such as glass slide) is not blurred, and an image in focus can be photographed, so that the accuracy of the screening method of the present invention using the stained part as an index can be enhanced.

When the confocal laser scanning microscope is used, it is preferable to use a fluorescence antibody method as the immunohistochemical staining method.

[0039] Subsequently, a range randomly selected from the photographed image, the range being within the part where the stratum corneum cells have been transferred, is expanded, and converted into a black and white image (binary image).

From the resulting image, area values of the stained part and/or the unstained part, or ratios (%) of the area of the stained part and/or the area of the unstained part are calculated (area simply referred to hereinafter includes both the area value and the area ratio).

[0040] The aforesaid image processing and calculation of area can be carried out using image analysis software.

An example of the image analysis software is "Image J (National Institutes of Health, NIH)".

[0041] The area of the stained part is preferably an average value of areas calculated from a plurality of ranges randomly selected.

By using an average value of areas of a plurality of places as an index, more accurate screening can be carried out.

[0042] Subsequently, the evaluation step S40 of evaluating an effect of the search target ingredient on the increase or decrease of the adhesive protein using the area obtained in the step S30 as an index is carried out.

[0043] In Figure 3, a flowchart illustrating the step S40 in detail is shown. The evaluation step in one

embodiment in which the area of the stained part of the screening sample and the area of the stained part of the screening control sample are compared will be described with reference to Figure 3.

[0044] In a step S41, an area A_t of the stained part of the screening sample and an area A_c of the stained part of the screening sample are compared.

The comparison between the areas may be a comparison between absolute values of areas, or may be a comparison between ratios of areas when assuming that the area of the screening control sample is 100%.

When a plurality of screening samples is evaluated, the comparison is preferably carried out assuming that the area of the screening control sample is 100%.

[0045] When the area A_t is smaller than the area A_c (area $A_t < \text{area } A_c$), a transition to a step S42 is made, and the search target ingredient having been added to the screening sample is evaluated as an ingredient having an action to decrease the amount of adhesive protein.

[0046] On the other hand, when the area A_t is the same as the area A_c or the area A_t is larger than the area A_c (Area $A_t \leq \text{Area } A_c$), a transition to a step S43 is made, and the search target ingredient having been added to the screening sample is evaluated as an ingredient having an action to decrease the amount of the adhesive protein.

[0047] The evaluation step according to one embodiment has been described with reference to Figure 3, but the

evaluation step in the screening method of the present invention is not particularly limited as long as the evaluation is an evaluation of an effect on the increase or decrease of adhesive protein using the area of the stained part of the screening sample as an index.

[0048] For example, when an ingredient to increase the amount of adhesive protein is intended to be screened, whether "area At > area Ac" is judged in the step S41, and if the result is "yes", in the step S42, the ingredient may be evaluated as an "ingredient having an action to increase the amount of adhesive protein".

Alternatively, in the step S42, the ingredient may be evaluated as an "ingredient having an action to decrease the amount of adhesive protein", and in the step S43, the ingredient may be evaluated as an "ingredient having an action to increase the amount of adhesive protein".

[0049] In another embodiment, the evaluation step may be a step of preparing a plurality of screening samples different in concentration of the search target ingredient and evaluating them, as described in the step S20.

In the present embodiment, when the areas in the plurality of the screening samples are compared and there is a specific concentration that becomes a threshold for the exhibition of a prominent effect in the action to decrease the amount of adhesive protein or the action to

increase the amount thereof, an evaluation that "the search target ingredient has a prominent action to decrease or increase the amount of adhesive protein at the specific concentration or more" can be made.

Regarding the ingredient that does not exhibit the above action unless the concentration is set to the specific concentration or more, an evaluation that "the search target ingredient exhibits an action to decrease or to increase the amount of adhesive protein at the specific concentration or more" can be made.

[0050] The screening method of the present invention is preferably used for screening of an ingredient that is used for improvement and/or prevention of sensitive skin.

Here, the "sensitive skin" in the present specification refers to skin quality having been decreased in resistance (having been decreased in skin stimulation value) to external stimuli (dryness, ultraviolet ray, application of compound).

[0051] More specifically, the "sensitive skin" in the present specification refers to skin quality presented by (1) and/or (2).

[0052] (1) "Skin that reacts to substances, such as medicinal external preparations, cosmetics, plants, ultraviolet rays and metals, and is prone to skin troubles. Skin that is sensitive to allergens (pollens, perfumes, etc.) or stimulating substances (alcohols, etc.)."

(2) "Skin that is temporarily prone to skin troubles with stimulating substances under circumstances of sleep deprivation, overwork, menstruation, change of seasons, psychological stress, etc."

[0053] The expression "for improvement and/or prevention of sensitive skin" in the present specification refers to improvement of skin quality having been decreased in resistance to external stimuli (having been decreased in skin stimulation threshold), and/or prevention of decrease in resistance to external stimuli (decrease in skin stimulation threshold) in the skin quality.

[0054] The screening method of the present invention is preferably used for screening of an ingredient having an intercellular adhesion inhibitory action for human epidermal keratinocytes.

In the present specification, the term "intercellular adhesion" refers to stratum corneum adhesion dysfunction (advance of stratification of stratum corneum due to increase of cell adhesiveness) caused by increase of desmosome proteins (protein group containing desmoglein) (if necessary, see Japanese Patent No. 4197194, Japanese Patent No. 4002635).

In the present specification, the concept of "intercellular adhesion inhibition" includes both of prevention and improvement of intercellular adhesion.

[0055] Here, by the inhibition of intercellular adhesion of human epidermal keratinocytes, stratification of

stratum corneum is inhibited, and an action to inhibit stratum corneum multilayer exfoliation can be obtained.

That is to say, the screening method of the present invention can be used for screening of an ingredient having an action to prevent or improve stratum corneum multilayer exfoliation.

In the present specification, the term "stratum corneum multilayer exfoliation" refers to exfoliation of stratum corneum that is in an overlapped state of a plurality of layers, from the skin surface. Whether the skin is in the state of stratum corneum multilayer exfoliation can be confirmed by collecting stratum corneum by a tape stripping method, staining the collected stratum corneum and observing it.

[0056] As shown in the examples described later, desmoglein 1 is present in a large amount at the place where the skin brightness is low, and lowering of skin brightness is thought to be due to stratification of stratum corneum.

In the light of the above knowledge, it can be said that the ingredient having an action to decrease desmoglein is an ingredient having a skin brightness improving action.

That is to say, the screening method of the present invention can be used for screening of an ingredient having a skin brightness improving action.

Here, in the present specification, the term "skin brightness improvement" refers to reduction of difference in brightness between the normal skin and the skin having been decreased in brightness due to the presence of excessive desmoglein.

[0057] As shown in the examples described later, the ingredient having a desmoglein decrease action has an action to improve skin condition to give clearer skin. That is to say, the screening method of the present invention can be used for screening of an ingredient having a skin clearness improving action.

[0058] As shown in the examples described later, desmoglein 1 is present in a large amount at the place where the skin hardness is high, and high skin hardness is thought to be due to stratification of stratum corneum.

Based on the above knowledge, by decreasing desmoglein, a skin softening effect is obtained. That is to say, the screening method of the present invention can be used for screening of an ingredient having a skin softening action.

[0059] Here, by softening the skin, penetration of other medicinal ingredients can be efficiently carried out. On that account, the screening method of the present invention can be used for screening of an ingredient having an action to allow medicinal ingredients such as a whitening agent to penetrate the skin more efficiently.

Examples

[0060] [Example 1]

<Stratum corneum cell sample preparation step>

Stratum corneum having been collected from the outside human forearm by a cellophane tape was transferred onto a glass slide ("MAS COAT", manufactured by Matsunami Glass Ind., Ltd.), and the glass slide was immersed in xylene overnight.

Subsequently, the glass slide was cleaned with xylene for 10 minutes twice and then cleaned with PBS.

[0061] <Preparation of search target ingredient>

A wild thyme extract (manufactured by Ichimaru Pharcos Co., ltd., terrestrial part extract of wild thyme (*T. serpyllum*)) and a *Rosa roxburghii* extract (manufactured by Maruzen Pharmaceutical Co., Ltd., fruit extract of *Rosa roxburghii*) were each diluted with PBS so that the concentrations might become 0.1 mass%, 0.05 mass%, 0.025 mass%, and 0.0125 mass%, thereby obtaining search target ingredients.

<Preparation of screening sample>

The stratum corneum cells were immersed in each of the search target ingredients overnight, thereby obtaining samples. As a control sample, stratum corneum cells that had not been immersed in the search target ingredient were prepared.

[0062] <Immunohistochemical staining test>

The samples were each cleaned with PBS and then incubated in a 4% PFA-phosphate buffer solution at room temperature for 15 minutes.

Subsequently, the samples were each cleaned with PBS and then incubated in a 20% Block Ace (manufactured by DS Biopharma Limited) aqueous solution at room temperature for 1 hour.

To each of the samples, a primary antibody (Anti-Desmoglein 1, Mouse-Mono) was added, and in a moist chamber, each of the samples was incubated at room temperature for 2 hours.

Each of the samples was cleaned with PBS for 5 minutes three times, and then, to each of the samples, a secondary antibody (Alexa Fluor@488 Goat Anti-mouse IgG) was added, and in a moist chamber, each of the samples was incubated at room temperature for 1 hour.

Each of the samples was cleaned with PBS for 5 minutes three times, then cleaned with distilled water twice, and then sealed using Fluoromount-G (manufactured by Southern Biotechnology Associates, Inc.), thereby obtaining a plurality of screening samples and a screening control sample.

[0063] <Image analysis>

The screening samples and the screening control sample were each photographed using a confocal laser scanning microscope to obtain images for analysis (Figure 4).

Using image analysis software (Image J), ranges of arbitrary 4 sites in each of the images for analysis were expanded and converted into black and white images, then areas of the stained parts and the unstained parts were calculated, and an average value was calculated.

Assuming that the area of the stained part of the screening control sample was 100%, the ratio of the area of the stained part in each of the screening samples was calculated (average value of 4 sites). The results are shown in Table 1 and Figure 5.

[0064] [Table 1]

Sample	Average	SD
No addition (control sample)	100	10.98127
0.1% Wild thyme	34.46502	14.89497
0.05% Wild thyme	85.26024	4.507292
0.025% Wild thyme	87.75052	7.99582
0.0125% Wild thyme	98.43656	10.21772
0.1% Rosa roxburghii extract BG	9.262649	8.040511
0.05% Rosa roxburghii extract BG	72.48284	14.28212
0.025% Rosa roxburghii extract BG	74.37402	9.343535
0.0125% Rosa roxburghii extract BG	70.43013	8.672259

[0065] As shown in Figure 4 and Figure 5, it can be seen that the screening samples in which the wild thyme extract and the Rosa roxburghii extract had been added were decreased in the expression level of desmoglein 1 as compared with the screening control sample.

From this result, the wild thyme extract and the Rosa roxburghii extract can be evaluated as ingredients having an action to decrease the amount of desmoglein 1.

[0066] Moreover, it can be seen that the screening sample having an added wild thyme extract concentration of 0.1 mass% was decreased in the area of the stained part by

about 2.5 times to 2.8 times as compared with the screening samples having the concentrations of 0.05, 0.025, and 0.0125 mass%.

[0067] Similarly to the above, it can be seen that the screening sample having an added *Rosa roxburghii* extract concentration of 0.1 mass% was decreased in the area of the stained part by 7.6 to 8.0 times as compared with the screening samples having the concentrations of 0.05, 0.025, and 0.0125 mass%.

[0068] From this, the wild thyme extract and the *Rosa roxburghii* extract can be evaluated as having a prominent action to decrease the amount of desmoglein 1 when the concentration thereof is 0.1 mass% or more.

[0069] [Example 2]

<Stratum corneum cell sample preparation step>

Stratum corneum cell samples were prepared in the same manner as in Example 1.

[0070] <Preparation of search target ingredient>

An *Althaea officinalis* extract (manufactured by Jurlique International Pty Ltd., root extract of *Althaea officinalis*) was diluted with PBS so that the concentration might become 0.25 mass%, thereby obtaining a search target ingredient.

[0071] <Preparation of screening sample>

The stratum corneum cells were immersed in the search target ingredient overnight, thereby obtaining a sample. As a control sample, stratum corneum cells that

had not been immersed in the search target ingredient were prepared.

[0072] <Immunohistochemical staining test>

A screening sample and a screening control sample were obtained in the same manner as in Example 1.

[0073] <Image analysis>

In the same manner as in Example 1, images for analysis of the screening sample and the screening control sample were photographed, and assuming that the area of the stained part of the screening control sample was 100%, the ratio of the area of the stained part in the screening sample was calculated. The results are shown in Table 2, Figure 6 and Figure 7.

[0074] [Table 2]

Test sample	Expression level of desmoglein 1 (%)
No addition	100
0.25 mass% Althaea officinalis extract	72.09

[0075] As shown in Figure 6 and Figure 7, it can be seen that the screening sample in which the Althaea officinalis extract had been added was decreased in the expression level of desmoglein 1 as compared with the screening control sample.

From this result, the Althaea officinalis extract can be evaluated as an ingredient having an action to decrease the amount of desmoglein 1.

[0076] [Example 3]

Hereinafter, test results to support a relationship between the difference in skin brightness and the abundance of desmoglein 1, and a relationship between the abundance of desmoglein 1 and the skin hardness are shown.

[0077] (1) Test subject and measurement site

In the present example, 20 females between the ages of 40 and 52 were selected as test subjects.

In the present example, on each test subject's face, a site where decrease in brightness had been able to be confirmed by visual observation (see blackish part, site A in Figure 13) and a site in normal state (see site B in Figure 13) were selected as measurement sites.

[0078] (2) Measurement

(2-1) Measurement of L* value, a* value and b* value

To the aforesaid measurement sites, a spectrophotometer was applied, and the sites were each subjected to measurement 5 times, thereby measuring L* value, a* value and b* value.

The results are shown in Figure 8 to Figure 10.

[0079] Regarding the L* value, the site where decrease in brightness had been able to be confirmed by visual observation (see blackish part, site A in Figure 13) exhibited a significantly lower value than the site in normal state (see site B in Figure 13). That is to say, it was able to be confirmed that the site where decrease in brightness had been able to be confirmed by visual observation (see blackish part, site A in Figure 13) was

darker than the site in normal state (see site B in Figure 13).

[0080] Regarding the a^* value, the site where decrease in brightness had been able to be confirmed by visual observation (see blackish part, site A in Figure 13) exhibited a significantly higher value than the site in normal state (see site B in Figure 13). That is to say, it was able to be confirmed that the site where decrease in brightness had been able to be confirmed by visual observation (see blackish part, site A in Figure 13) was more reddish than the site in normal state (see site B in Figure 13).

[0081] Regarding the b^* value, the site where decrease in brightness had been able to be confirmed by visual observation (see blackish part, site A in Figure 13) exhibited a significantly higher value than the site in normal state (see site B in Figure 13). That is to say, it was able to be confirmed that the site where decrease in brightness had been able to be confirmed by visual observation (see blackish part, site A in Figure 13) was more yellowish than the site in normal state (see site B in Figure 13).

[0082] (2-2) Measurement of hardness

To each of the measurement sites having been subjected to the above test (2-1), an indentation hardness meter was pressed, and each of the sites was

subjected to measurement 5 times, thereby measuring hardness.

The results are shown in Figure 11.

[0083] Regarding the hardness, the site where decrease in brightness had been able to be confirmed by visual observation (see blackish part, site A in Figure 13) exhibited a significantly lower value than the site in normal state (see site B in Figure 13). That is to say, it was able to be confirmed that the site where decrease in brightness had been able to be confirmed by visual observation (see blackish part, site A in Figure 13) was harder than the site in normal state (see site B in Figure 13).

[0084] (2-3) Staining of desmoglein 1 (Dsg1)

The stratum corneum cells of each of the measurement sites having been subjected to the above tests (2-1) and (2-2) were collected by a tape stripping method and stuck to a glass slide.

[0085] The glass slide to which the stratum corneum cells had been stuck was immersed in xylene overnight.

After the immersion, the glass slide was subjected to cleaning and air drying, and then it was allowed to stand still in a 4% paraformaldehyde-phosphate buffer solution for 15 minutes under the conditions of room temperature.

[0086] In 15 minutes, the stratum corneum cells were cleaned with PBS, and immersed in a 0.5% Triton X

(manufactured by Nacalai Tesque, Inc.)/PBS solution for 5 minutes under the conditions of room temperature.

[0087] After the immersion, the stratum corneum cells were cleaned and allowed to stand still in a Block Ace aqueous solution (DS Biopharma Limited UK-B80) for 1 hour under the conditions of room temperature.

[0088] After the still standing, the stratum corneum cells were framed with Liquid Blocker (manufactured by Daido Sangyo Co., Ltd.), then a primary antibody was added thereto, and the stratum corneum cells were allowed to stand still in a moist chamber for 2 hours under the conditions of room temperature.

As the primary antibody, Anti Desmoglein 1, Mouse Mono (Dsg1 P23) Progen, 651110 undiluted solution (IgG concentration: 10-15 g/mL) was used.

[0089] After the still standing, the stratum corneum cells were cleaned using PBS, then a secondary antibody was added thereto, and the stratum corneum cells were allowed to stand still in a moist chamber for 1 hour under the conditions of room temperature.

As the secondary antibody, a PBS 200-fold dilute solution of Alexa Fluor@488 Goat Anti mouse IgG Invitrogen, A 11001 was used.

[0090] After the still standing, the stratum corneum cells were cleaned, then sealed with Fluoromout-G (manufactured by Southern Biotechnology Associates, Inc.), and dried for 30 minutes at room temperature.

[0091] After the drying, the cover glass was fixed by manicure and dried, and then the stratum corneum cells were subjected to observation by a confocal laser scanning microscope (Nikon A1) at x20 magnification (photographing).

The results are shown in Figure 12.

[0092] Regarding the amount of desmoglein 1 (Dsg1), the site where decrease in brightness had been able to be confirmed by visual observation (see blackish part, site A in Figure 13) exhibited a significantly higher value than the site in normal state (see site B in Figure 13). That is to say, it was able to be confirmed that the amount of desmoglein 1 (Dsg1) in the site where decrease in brightness had been able to be confirmed by visual observation (see blackish part, site A in Figure 13) was larger than that in the site in normal state (see site B in Figure 13).

[0093] (3) Consideration

As shown in Figure 8 to Figure 10 and Figure 12, it has been proved that desmoglein 1 is present in a large amount at the place where the skin brightness is low. Here, lowering of the skin brightness is thought to be due to dense overlapping of stratum corneum (stratification of stratum corneum) because of low pH of the skin and low activity of serine protease that decomposes desmoglein 1.

[0094] As previously described, the active ingredient of the present invention exhibits a desmoglein decrease action.

In the light of the above knowledge, it has been proved that the active ingredient of the present invention exhibits a skin brightness improving action attributable to decrease of desmoglein 1.

[0095] As shown in Figure 11 and Figure 12, it has been proved that desmoglein 1 is present in a large amount at the place where the skin hardness is high. Here, the cause of high skin hardness is thought to be due to dense overlapping of stratum corneum (stratification of stratum corneum) because of low pH of the skin and low activity of serine protease that decomposes desmoglein 1.

[0096] Based on the above knowledge, it has been proved that by decreasing desmoglein 1, the skin is softened.

As previously described, the active ingredient of the present invention exhibits a desmoglein decrease action.

That is to say, it has been proved that the active ingredient of the present invention exhibits a skin softening action attributable to decrease of desmoglein 1.

[0097] [Example 4]

Hereinafter, verification results of the multilayer exfoliation inhibitory action of a composition containing an ingredient having a desmoglein decrease action are shown.

(1) Preparation of formulation

According to the composition described in Table 3, a cosmetic of Preparation Example 1 containing a Rosa roxburghii extract was prepared.

[0098] [Table 3]

Ingredient	Preparation Example 1
Fatty acid	40
Glycerol	19.2
Diglycerol	3.5
Carboxymethyl cellulose sodium salt	0.4
Acrylic acid-diallyl dimethyl ammonium chloride-acrylamide copolymer solution	2
Diallyl dimethyl ammonium chloride-acrylamide copolymer solution	4.5
Potassium hydroxide	8.5
Alkyl glycoside	1.5
Rosa roxburghii extract	0.4
Water	20
Total	100

[0099] (2) Test subject and target site

In the present example, five females were selected as test subjects, and to the right and left medial sides of forearms (n=10) of the test subjects, the cosmetic of Preparation Example 1 was applied twice a day (morning and night), and this was continued for 10 days.

[0100] (3) Verification of desmoglein decrease action

At each stage before use and after use (after ten consecutive days) of the cosmetic of Preparation Example 1, stratum corneum of the right and left medial sides of forearms of each test subject was collected in accordance

with the method described in "<Stratum corneum cell sample preparation step>" of Example 1, thereby preparing stratum corneum cell samples. Subsequently, in accordance with the method described in "<Immunohistochemical staining test>" of Example 1, desmoglein 1 in each of the stratum corneum cell samples was stained, then using a confocal laser scanning microscope, images for analysis before and after use of Preparation Example 1 were photographed, and confirmation of fluorescence (staining) was carried out.

[0101] Assuming that the area of the whole image for analysis was 100%, the area of the fluorescence part was calculated. The results are shown in Figure 14 and Figure 15. In Figure 15, the symbol * means $p < 0.05$.

[0102] As shown in Figure 14 and Figure 15, it was able to be confirmed that by continuously using the cosmetic of Preparation Example 1 containing a *Rosa roxburghii* extract, the amount of desmoglein 1 was decreased.

[0103] (4) Verification of multilayer exfoliation inhibitory action

Before use and after use of the cosmetic of Preparation Example 1, stratum corneum of the right and left medial sides of forearms of each test subject was collected in accordance with the method described in "<Stratum corneum cell sample preparation step>" of Example 1, thereby preparing stratum corneum cell samples. Subsequently, each of the stratum corneum cell samples

was stained in a conventional way using gentian violet and brilliant green, and it was observed by an optical microscope.

Typical drawings of the results are shown in Figure 16.

[0104] As shown in Figure 16, in the optical microscope image of the stratum corneum cells before use of Preparation Example 1, a plurality of deep color parts is present. This deep color part is a part where the stratum corneum cells suffered multilayer exfoliation due to the tape stripping method.

On the other hand, the stratum corneum cell samples having been collected after continuous use of Preparation Example 1 for 10 days were faintly stained for the most part as compared with the stratum corneum cell samples having been collected before use. This faintly stained part is a part where only one layer of the stratum corneum cells was exfoliated, that is, this means that multilayer exfoliation did not occur.

As described above, it was able to be confirmed that by using the cosmetic of Preparation Example 1 containing an ingredient having a desmoglein decrease action, multilayer exfoliation was improved.

[0105] [Example 5]

Hereinafter, desmoglein decrease ability of a composition containing an ingredient having a desmoglein decrease action was examined.

[0106] (1) Preparation of composition

A cosmetic (emulsion) containing a wild thyme extract was prepared in accordance with the formulation shown in the following Table 4. That is to say, the ingredients (a), (b) and (c) were each heated to 70°C, then the ingredient (c) was added to the ingredient (b) to perform neutralization, then while stirring the mixture, the ingredient (a) was slowly added thereto to perform emulsification, and the resulting emulsion was homogenized using a homogenizer and then cooled while stirring, thereby obtaining an emulsion of Preparation Example 2.

[0107] [Table 4]

Ingredient		Preparation Example 2
(a)	Squalane	10
	Sorbitan sesquistearate	2
	Wild thyme extract	0.1
	Butyl paraben	0.1
(b)	1,3-Butanediol	5
	Xanthan gum	0.1
	Acrylic acid-alkyl methacrylate (C10-30)	0.4
	Methyl paraben	0.1
	Water	50
(c)	Potassium hydroxide	0.2
	Water	32
Total		100

[0108] (2) Test subject and measurement site

In the present example, 20 females between the ages of 40 and 52 were selected as test subjects.

In the present example, on the test subject's faces, sites (92 sites) where spots were present were selected as measurement sites.

To the sites of each test subject where spots were present, the emulsion of Preparation Example 1 was applied twice a day, and this was continued for 12 weeks.

[0109] (3) Measurement

Before use of the emulsion of the preparation example, after a lapse of 6 weeks of continuous use, and after a lapse of 12 weeks, stratum corneum cells at the test subject's site where spots were present were collected in accordance with the method described in "<Stratum corneum cell sample preparation step>" of Example 1, thereby preparing stratum corneum cell samples. Subsequently, in accordance with the method described in "<Immunohistochemical staining test>" of Example 1, desmoglein 1 in each of the stratum corneum cell samples was stained, then using a confocal laser scanning microscope, images for analysis before and after use of Preparation Example 2 were photographed, and confirmation of fluorescence (staining) was carried out.

[0110] Assuming that the area of the whole image for analysis was 100%, the area of the fluorescence part was selected. The results are shown in Table 5, Table 6, Figure 17 (typical), and Figures 18 and 19. As the data after a lapse of 12 weeks of use of the emulsion, data at 80 sites of the 93 spotted sites were aggregated, and

they were compared with the data at the same sites before use of the emulsion. In Figure 18, the symbol ** means $p < 0.01$, and in Figure 19, the symbol *** means $p < 0.001$.

[0111] [Table 5]

Amount of desmoglein 1 (%)	Before use	Continuous use for 6 weeks
Average	23.9	20.9
Standard deviation	9.42	9.68
The number of samples	n=92	n=92

[0112] [Table 6]

Amount of desmoglein 1 (%)	Before use	Continuous use for 12 weeks
Average	23.2	10.6
Standard deviation	7.88	9.62
The number of samples	n=80	n=80

[0113] As shown in Tables 5 and 6, and Figures 17 to 19, it was able to be confirmed that by continuously using the emulsion containing a wild thyme extract, the amount of desmoglein 1 at the spotted site was significantly decreased.

Industrial Applicability

[0114] The present invention is applicable to analytical methods related to the amount of adhesive protein.

Claims

[Claim 1]

A method for screening an ingredient that affects the increase or decrease of adhesive protein in stratum corneum cells, comprising:

- a collecting step of collecting stratum corneum cells using a tape stripping method,

- a transfer step of transferring stratum corneum onto a glass slide having a positively charged surface,

- a cleaning step of cleaning the glass slide,

- a preparation step of adding a search target ingredient to the glass slide and staining adhesive protein by an immunohistochemical staining method to prepare a screening sample,

- an image acquiring step of photographing an arbitrary place of the screening sample to acquire an image for analysis, and

- an evaluation step of evaluating an effect of the search target ingredient on the increase or decrease of the adhesive protein using, as an index, an area of a stained part in the image for analysis.

[Claim 2]

The screening method according to claim 1, wherein in the preparation step, a screening control sample is prepared without adding the search target ingredient, and

the evaluation step is a step of evaluating the search target ingredient as having an action to decrease the amount of adhesive protein when an area of a stained part of the screening sample and an area of a stained part of the screening control sample are compared with each other to find that the area of the stained part of the screening sample is smaller.

[Claim 3]

The screening method according to claim 1, wherein in the preparation step, a plurality of screening samples identical in type of the search target ingredient and different in concentration thereof is prepared, and

the evaluation step is a step of evaluating an optimum concentration in the action to decrease the amount of adhesive protein by comparing areas of stained parts of the plurality of the screening samples.

[Claim 4]

The screening method according to any one of claims 1 to 3, wherein the adhesive protein is desmoglein 1.

[Claim 5]

The screening method according to any one of claims 1 to 4, wherein the glass slide is a glass slide having NH_3^+ arranged on a surface thereof.

[Claim 6]

The screening method according to any one of claims 1 to 5, wherein the cleaning step is a step of immersing the glass slide in xylene.

[claim 7]

A method for preparing a stratum corneum cell sample using a tape stripping method, comprising:

a transfer step of transferring stratum corneum onto a glass slide having a positively charged surface, and

a cleaning step of cleaning the glass slide.

[Claim 8]

The preparation method according to claim 7, wherein the glass slide is a glass slide having NH_3^+ arranged on a surface thereof.