Studies on the effects of unsaturated fatty acids on epidermal keratinocytes

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Contents

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February, 2019

Yuji Katsuta

Preface

The skin covers the entire external surface of the human body. It is the largest human organ, occupying an area of about 1.6 m^2 in adults and accounts for about 16% of body weight. It serves many important functions, including preventing water evaporation from the body, protecting the body against trauma, regulating body temperature, and sensing stimuli. The skin consists of three layers: the epidermis, the dermis, and the subcutaneous fat, in order from the surface. In addition to the three layers, it contains cutaneous appendages such as hair, nails, sebaceous glands and sweat glands. Nerve fibers, blood and lymphoid vessels, along with immune cells such as mast cells and Langerhans cells are also present in the skin. In this way, the structure of skin is complicated, and various kinds of cells function in a mutually dependent fashion. The schematic diagram of skin is shown in figure 1.

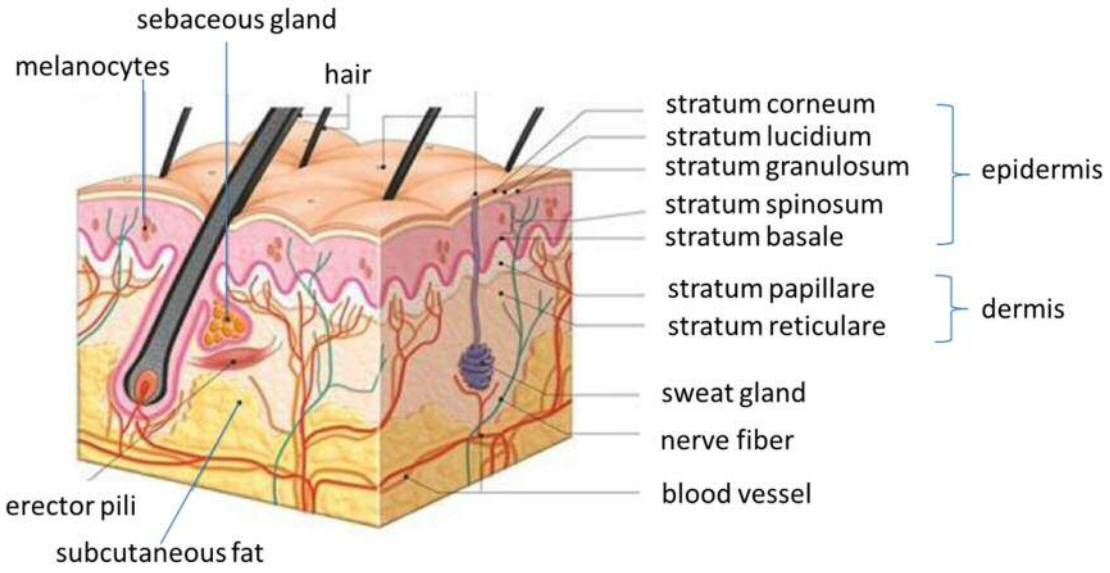


Figure 1. Schematic diagram of the skin

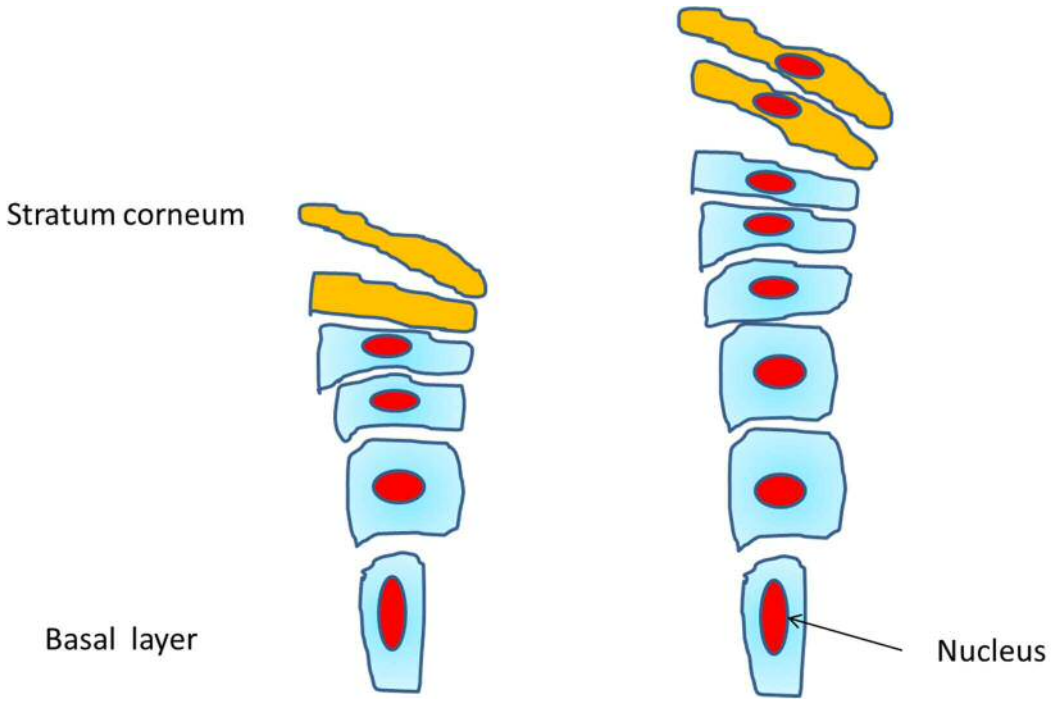
The source: Katei Igaku Daizenka (6th ed) Houken Corp.

The epidermis consists of keratinocytes (95%), but also contains melanocytes, Langerhans cells, Merkel cells, and inflammatory cells. The epidermis is composed of four or five layers, i.e. stratum corneum, stratum lucidium (only in palms and soles), stratum granulosum, stratum spinosum, and stratum basale. The stratum basale attaches to the basement membrane, which connects the epidermis and the dermis. Keratinocytes proliferate only in the stratum basale in normal conditions. After detaching from the basement membrane, keratinocytes start differentiation and move into the upper layers. In the stratum spinosum, keratinocytes start producing lamellar bodies enriched in polar lipids, glycosphingolipids, free sterols,

phospholipids and catabolic enzymes. In the stratum granulosum, keratinocytes become flat, and nuclei and organelles disappear. Lipids in the lamellar bodies are released into the extracellular space through exocytosis to form intercellular lipids. The stratum corneum, the outermost layer of the epidermis, is composed of dead cells, which are called corneocytes. This layer is composed of 15–20 layers of flattened cells with no nuclei or cell organelles. Their cytoplasm shows filamentous keratin. These corneocytes are embedded in a lipid matrix composed of ceramides, cholesterol, and fatty acids. The stratum corneum functions to form a barrier to protect underlying tissue from dehydration and infection. Then, the outermost corneocytes shed from the surface of the stratum corneum and this process is called as desquamation.

Differentiation of keratinocytes is very important for the functions of the epidermis. As the epidermis is on the outermost surface of a body, it is exposed to surrounding environment. These environmental factors such as dryness, ultraviolet rays, and allergic substances affect the epidermal functions. They induce abnormal epidermal differentiation, which leads to

impaired barrier function, and dry and scaly skin. Corneocytes which fail to lose their nuclei are often seen in these damaged epidermal conditions. This situation with the retention of nuclei in the stratum corneum is called parakeratosis and is used as an index of abnormal epidermal differentiation. Parakeratosis also occurs with pathological conditions such as atopic dermatitis and psoriasis.



(a) Normal differentiation (b) Abnormal differentiation

Figure 2. Schematic diagram of the epidermis. (a): Normally differentiated epidermis. (b): Abnormally differentiated epidermis with parakeratosis.

Sebum is produced in sebaceous glands and secreted to the skin's surface through hair follicle ducts. Sebum is thought to have a moisturizing effect and an antibacterial effect, but its function is not well understood. In humans, sebaceous glands are localized on all parts of the skin except for palms and soles. They present in the greatest number on the face and scalp. There are 200 sebaceous glands per square centimeter in the face and scalp, but only about 10 to 30 in the shin. Secretion of sebum is most abundant in the late teens to 20s, and then starts to decrease gradually in the 30s in women and in the 50s in men.

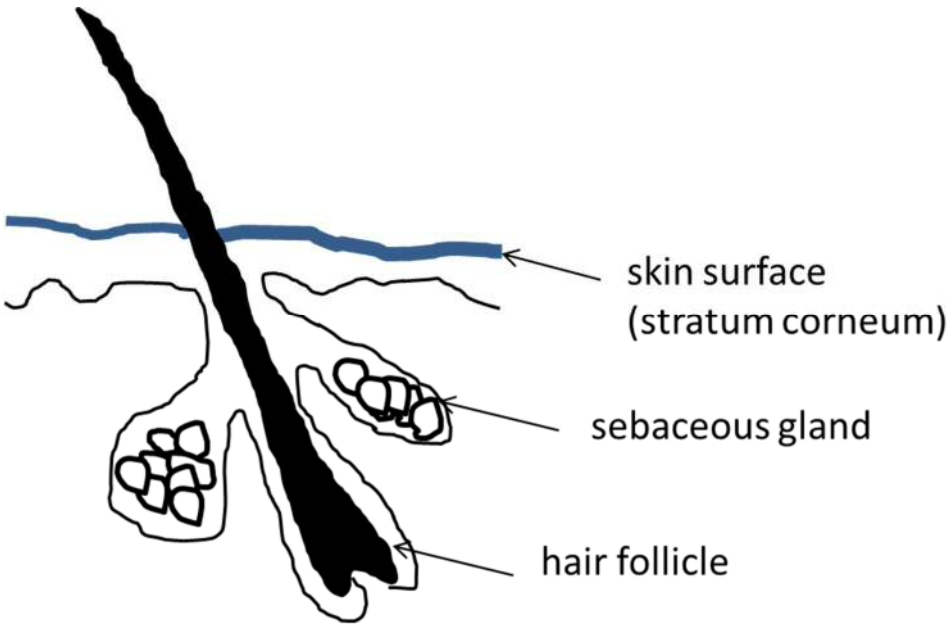


Figure 3. Schematic diagram of hair follicle and sebaceous gland

An appropriate amount of sebum is thought to have a moisturizing effect on the skin, but excessive sebum has a bad influence on the skin. A typical example is acne vulgaris. Acne vulgaris is a type of dermatitis often seen in adolescents. The first step of the formation of acne vulgaris is abnormal epidermal differentiation in follicular ducts. As acne vulgaris is observed in those with excessive sebum secretion, excessive sebum is regarded to be one the cause of abnormal differentiation.

Seborrheic dermatitis is another dermatitis related to excessive sebum. The symptoms of this dermatitis include scaly, greasy, itchy and red skin, and the differentiation of the epidermis is abnormal. In addition to acne vulgaris, conspicuous facial pores are also skin complications associated with excessive sebum.

Although excessive sebum is related to abnormal epidermal differentiation in some types of dermatitis, the mechanism involved in the abnormal epidermal differentiation caused by excessive sebum has not been clarified.

Human sebum is composed of wax esters, squalene, triglycerides, and fatty acids. Of these components, triglycerides and free fatty acids together account for about 57% of the total sebum [1]. The free fatty acids are released from triglycerides in the sebum by bacteria, such as *Propionibacterium acnes*, in the ducts of hair follicles and on the skin surface. Fatty acids in sebum are composed of saturated and unsaturated fatty acids with a carbon chain length of C8 to C20. Fatty acids with a carbon chain length of C14, C16, and C18 account for 80% of total fatty acids. C16 and C18 fatty acids are especially predominant, and each have saturated and unsaturated fatty acids. Saturated fatty acid of C18 (C18:0) is called stearic acid and that of (C16:0) is called palmitic acid. The major C18 monounsaturated acid is oleic acid (18:1Δ9). As for C16 monounsaturated acids, palmitoleic acid (16:1Δ9) and 16:1Δ7 are predominant in mouse sebum [2]. However, these C16 monounsaturated acids are not highly present in human sebum. Sapienic acid, (16:1Δ6) and 16:1Δ8 are the major C16 monounsaturated acids in human sebum [3]. Regarding the melting point, if the degree of unsaturation of fatty acids is the same, melting point increased as carbon chain length becomes longer. As degree of unsaturation becomes higher, and carbon chain

length becomes shorter, melting point decreases. The structures of these main fatty acids in sebum are shown in Figure 4.

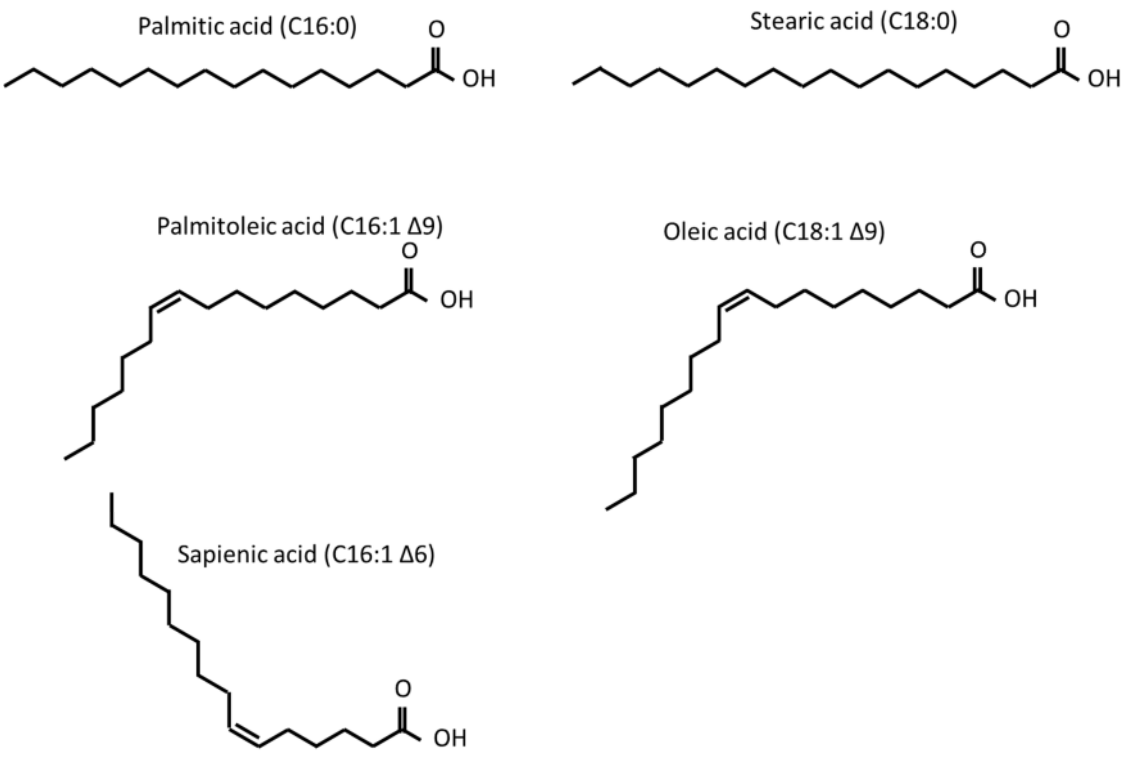


Figure 4. Structures of the main fatty acids in sebum

In this dissertation, for the purpose of clarifying the mechanism of abnormal epidermal differentiation due to excessive sebum, the effect of sebum components (especially unsaturated fatty acid) on epidermal differentiation were examined. In Chapter 1, the relationship between the conspicuous facial pores and sebum components was examined. Like acne vulgaris, conspicuous facial pores have been suggested to be related to excessive sebum. However, the actual conditions were not clear. As a result of the human volunteer test performed in this study, conspicuous facial pores were confirmed to be related to sebum, especially to the ratio of unsaturated fatty acids. In Chapter 2, the effects of unsaturated fatty acids on the differentiation of the epidermis and the calcium influx into keratinocytes were examined. In Chapter 3, the mechanism of the skin damage from abnormal differentiation and the calcium influx by unsaturated fatty acids were investigated. We hypothesized that the ion channel receptors might be involved, and thus the effects of ion channel receptor antagonists were examined.

Abbreviations

a.u.	arbitrary unit
BSS	balanced salt solution
$[Ca^{2+}]_i$	intracellular calcium concentration
EGTA	ethylene glycol tetraacetic acid
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
IL-1α	Interleukin 1 alpha
NMDA	N-methyl-D-aspartic acid
PBS	Phosphate buffered saline
RT	room temperature
RT-PCR	reverse transcription - polymerase chain reaction
S.D.	standard deviation
S.E.	standard error
TEWL	trans-epidermal water loss
TNF-α	Tumor Necrosis Factor alpha
w/w	weight per weight

Lists of Publications

Main publications

[1] Yuji Katsuta, Toshii Iida, Shinji Inomata and Mitsuhiro Denda,

"Unsaturated fatty acids induce calcium influx into keratinocytes and cause abnormal differentiation of epidermis"

J Invest Dermatol. Vol.124, No. 5, pp.1008-1013 (2005)

[2] Yuji Katsuta, Kiyotaka Hasegawa, Toshii Iida, Shinji Inomata and Mitsuhiro Denda,

"Function of oleic acid on epidermal barrier and calcium influx into keratinocytes is associated with N-methyl D-aspartate-type glutamate receptors."

Br J Dermatol. Vol.160, No. 1, pp.69-74 (2009)

Subsidiary publications

[3] Yuji Katsuta, Yuzo Yoshida, Eriko Kawai, Yoshiyuki Kohno and Kenji Kitamura,

"Urokinase-type plasminogen activator is activated in stratum corneum after barrier disruption"

J Dermatol Sci. Vol.32, No. 1, pp.55-57 (2003)

[4] Yuji Katsuta, Yuzo Yoshida, Eriko Kawai, Masaru Suetsugu, Yoshiyuki Kohno, Shinji Inomata and Kenji Kitamura,

"trans-4-(Aminomethyl)cyclohexane carboxylic acid methylamide (t-AMCHA methylamide) inhibits the physical interaction between urokinase-type plasminogen activator and stratum corneum, and accelerates the recovery of barrier function."

J Dermatol Sci. Vol.40, No. 3, pp.218-220 (2005)

[5] Yuji Katsuta, Yuki Ogura, Shunsuke Iriyama, Paul F Goetinck, John F Klement, Jouni Uitto and Satoshi Amano,

"Fibulin-5 accelerates elastic fibre assembly in human skin fibroblasts."

Exp Dermatol. Vol.17, No. 10, pp.837-842 (2008)

Chapter 1.

Relationship between conspicuous facial pores and sebum components

Chapter 1. Relationship between conspicuous facial pores and sebum components

1.1. Abstract

Conspicuous facial pores are one of the most frequent skin problems. Although it is considered that conspicuous facial pores are related to excessive sebum, the characteristics of the facial pores have not been fully investigated. In order to further clarify the characteristics of the pores, we performed tests to determine the relationship between the degree of conspicuity of the facial pores and the skin condition in 59 female volunteers. It was revealed that females with conspicuous pores has a large amount of sebum. The analysis of the sebum components showed that the ratio of unsaturated fatty acids, such as oleic acid, is high in the conspicuous pore group. The group with conspicuous pores has a lower barrier function and more parakeratotic corneocytes, suggesting that epidermal differentiation is abnormal.

These results suggest that sebum secreted from facial pores damages the stratum corneum which directly surrounds the facial pore. We posited that, should this be the case, the stratum corneum around facial pores might be worse than that of the inter-follicular areas, which is away from the conspicuous facial pores. To address this issue, we next compared the stratum corneum around facial pores and the inter-follicular epidermis and found that the nucleated parakeratotic corneocytes are particularly abundant in the skin around the pores. Visualization of skin barrier function by topical application of fluorescein showed that the barrier function is deteriorated around the pore.

Taken together, these results suggest that unsaturated fatty acids in the sebum are the candidates that cause abnormal epidermal differentiation around the pores, and this abnormal differentiation may make the facial pores conspicuous.

1.2. Introduction

Pigmentation, wrinkles and sagging are all major skin problems that people want to solve. The sizes of the whitening, anti-wrinkle, and skin-tightening cosmetic markets are evidence for this fact. Conspicuity of the facial pores is also one of the most frequent skin problems. Compared to pigments, wrinkles and sagging, conspicuous facial pores are of greater concern for the younger generation, according to the women volunteers from our test. Conspicuous facial pores are suggested to be related to excessive sebum like acne vulgaris. However, the characteristics of pores are not clarified.

In acne vulgaris, the epidermis in the follicular duct is abnormally differentiated with inflammation. This abnormal epidermal differentiation made the skin around the pore bulge. On the other hand, the skin around the pore has a hollow cone shape in conspicuous facial pores. Although excessive sebum is considered to be one of the causes of both of acne vulgaris and conspicuous facial pores, the structure around the pores seems different. As conspicuous facial pores are not regarded as dermatitis, they have not been investigated much compared to acne vulgaris. The actual condition of conspicuous facial pores remained to be investigated.

In this chapter, the characteristics of facial pores were studied. First, the relationship between the degree of conspicuity of facial pores and the skin condition parameters of female volunteers was studied, since it was hypothesized that conspicuous facial pores might be related to excessive sebum and abnormal epidermal differentiation. Then, the physiology of the skin around the facial pores was investigated.

In the course of the skin physiology study, we visualized the skin barrier function using fluorescence staining. The stratum corneum located in the outermost layer of the living body functions as a barrier for mass transfer in and out of the living body. This function prevents the evaporation of water from inside the living body and prevents invasion from harmful substances, such as allergens from the outside. For the measurement of the barrier function, it is common to measure the trans-epidermal water loss (TEWL). Although this method is an excellent method that can evaluate the skin barrier function easily and quantitatively, it is impossible to evaluate the skin function in small areas like facial pores. Therefore, a method to observe the skin barrier function in small areas like facial pores was developed.

In the field of ophthalmology, the fluorescein staining method is widely

and clinically used as a method to detect corneal epithelial tissue damage [4]. When the cornea is damaged, fluorescein penetrates the cornea and the part of damaged cornea is dyed. This method is used as a diagnostic method for dry eyes. I thought that it could be applied as an evaluation method for the skin barrier function. Therefore, fluorescein sodium was applied to the skin and the penetration state of the fluorescent substance into the deep part of the stratum corneum was observed using a fluorescence video microscope. Using this method, we observed the deterioration of the stratum corneum barrier function around the pores non-invasively.

1.3. Materials and Methods

1.3.1. Materials

Oleic acid was purchased from Wako Pure Chemical (Osaka, Japan). Fluorescite Injection was purchased from Alcon Japan Ltd (Tokyo, Japan) and used as the fluorescein sodium salt.

1.3.2. Human volunteer test

A skin measurement test was conducted on 59 Japanese women volunteers in their 20s and 30s. Photographs of the face were taken to grade the conspicuous facial pores. Sebum was collected for component analysis (written in 1.3.3). Tape-stripped stratum corneum was collected to count parakeratotic corneocytes.

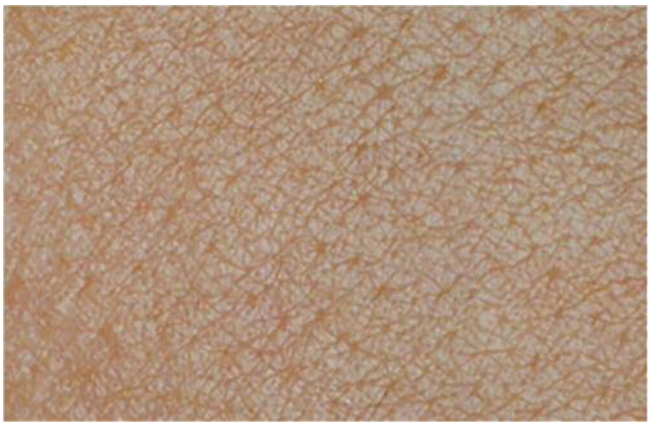
Transpepidermal water loss (TEWL), an indicator of the barrier function of stratum corneum, was measured using a TEWA meter TM210 (Courage + Khazaka, Cologne, Germany). TEWL is the loss of water that passes from inside a body through the epidermis to the surrounding atmosphere via diffusion and evaporation processes. The evaporation rare of water from the skin surface is expressed in g/hour/m^2.

Skin conductance (a parameter that indicates stratum corneum water content) was measured using a Skicon-200 (I.B.S. Company LTD., Hamamatsu, Japan). Skin conductance indicates the water contents in stratum corneum due to the fact that a substance which contains a large amount of water has a proportionately larger electric conductivity.

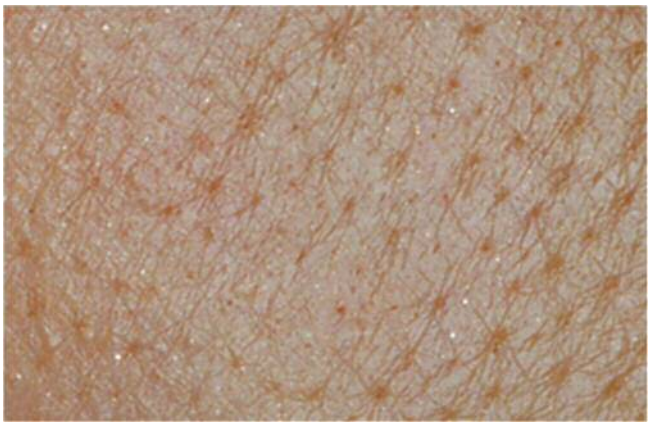
Grading the conspicuity of the facial pores was performed into three

groups, according to the degree of conspicuity of the pores from cheek photographs; (A) not conspicuous, (B) normal, (C) conspicuous (figure 5). The numbers of each group are A: n=11, B: n=22, C: n=18.

(A) Not conspicuous



(B) Normal



(C) Conspicuous

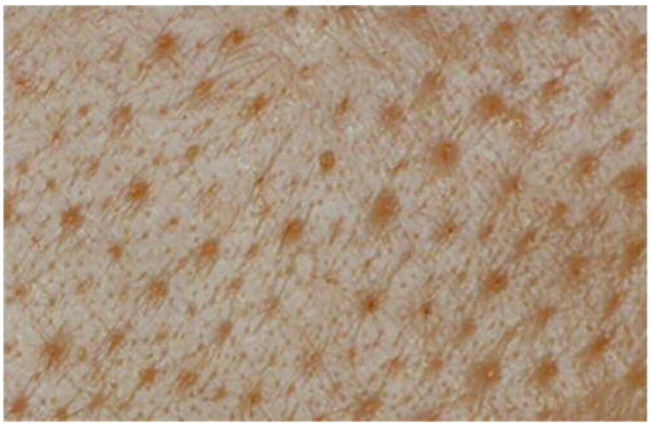


Figure 5. Grouping according to the degree of conspicuity of the pores in the cheek. Representative photographs are shown.

1.3.3. Analysis of sebum components

30 minutes after washing the face, Whatman filter paper of 2 cm × 2 cm was placed on the cheek for one minute, and the newly secreted sebum after the face washing was collected. Then the sebum components were extracted from the filter paper with acetone and were quantified using gas chromatography. The amounts of free fatty acid, squalene, wax, cholesterol, and triglyceride were quantified respectively, and their total amounts were taken as the total sebum content. For free fatty acids, the respective amounts of C16:0 (palmitic acid). C16:1 (sapienic acid or palmitoleic acid), C18:0 (stearic acid), C18:1 (oleic acid) were quantified. The amount of C16:0 + C18:0 was substituted with saturated fatty acids, and the amount of C18:1 + C16:1 was substituted with unsaturated fatty acids. In the ratio analysis, the data of eight volunteers was excluded because the total sebum amounts collected from them were less than 30μg and the ratio values were unreliable.

1.3.4. Detection of parakeratotic corneocytes in tape-stripped stratum corneum

The skin surface stratum corneum was collected using Carton Tape (Nichiban, Tokyo, Japan). A 10 μg per mL solution of Hoechst33342 (Sigma, St. Louis, Missouri) in phosphate-buffered saline (PBS) was applied to corneocytes attached to the adhesive tape and incubated for 30 minutes at RT, then washed with water. Parakeratotic corneocytes with nuclei were observed with a fluorescence microscope and the number of parakeratotic corneocytes in the area of a 35-mm slide film were counted. Sample numbers for this test do not match the total from the other experiments because of discounted candidate samples. (A: n=5, B: n=13, and C: n=8.)

1.3.5. *In vivo* non-invasive observation of the permeability of the stratum corneum on forearm skin using fluorescein sodium

Three hours of occlusive application of 30% solution of oleic acid in ethanol was performed on the forearms of male volunteers for two consecutive days. This treatment lowered the skin barrier function. A 0.1% aqueous solution of fluorescein sodium was applied occlusively for five minutes to skin treated

with oleic acid and the control intact skin. After the application, the skin surface was washed with water and then the surface layers of stratum corneum were removed by tape stripping twice. The fluorescence of the skin was observed using a fluorescence video microscope and fluorescence intensity in the image was quantified and compared with the TEWL value. TEWL was measured by using a Vapometer (Delfin Technologies, Finland).

1.3.6. *In vivo* non-invasive observation of the permeability of the stratum corneum on facial skin using fluorescein sodium

A 0.1% aqueous solution of fluorescein sodium was applied occlusively for one minute to the cheeks of male volunteers. After the application, the skin surface was washed with water and then the surface layers of stratum corneum were removed by tape stripping. The fluorescence of the skin was observed using a fluorescence video microscope. The application time was set shorter than the forearm treatment because the barrier function of facial skin is lower than that of the forearm.

1.3.7. Statistics

The results are expressed as the mean ± S.D. or S.E. In cases when only 2 groups were being examined, the statistical significance of the differences between them was determined by applying a two-tailed Student's *t*-test. In the case of more than 2 groups, the significance of differences was determined by means of Tukey–Kramer method. *$p<0.05$.

1.3.8. Ethics of human volunteer studies

Human volunteer tests were conducted under the approval of Shiseido Ethics Committee.

1.4. Results

1.4.1. Relationship between conspicuous facial pores and sebum

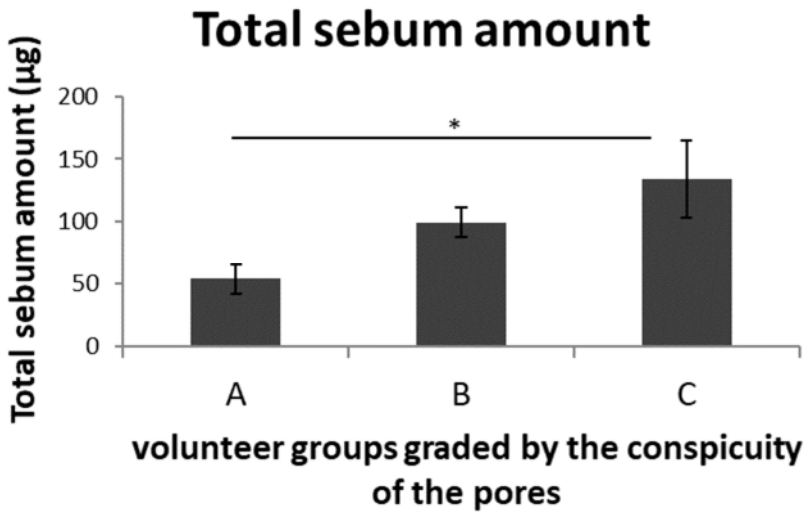
A skin measurement test was conducted on 59 Japanese women in their 20s and 30s and the volunteers were classified according to the degree of conspicuity of the facial pores into three groups (A: Not conspicuous, B: Normal, C: Conspicuous) by visual evaluation over the photographs of their

faces. Sebum secreted during the 30 minutes after the face washing was collected with filter paper and the sebum components were quantitated by gas chromatography. First, the total sebum amount was compared in each group. It was revealed that the total sebum amount increased as the pores became conspicuous (Figure 6a).

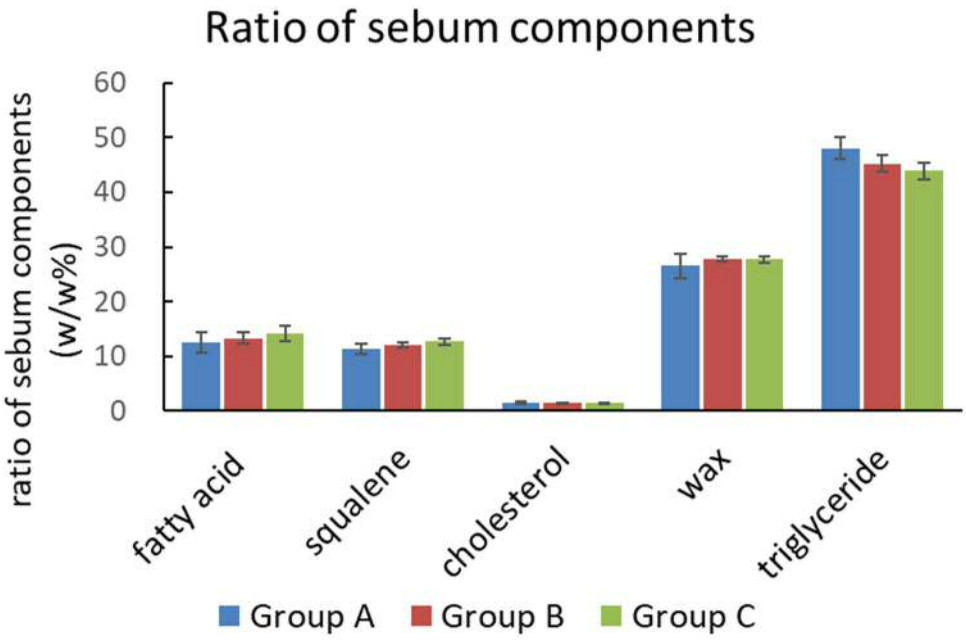
Next, the composition ratio of each sebum component was compared in each group. Comparisons of component ratios of free fatty acid, squalene, cholesterol, wax and triglyceride revealed that as the pores became more conspicuous, the ratio of triglyceride decreased, and the ratio of free fatty acid tended to increase, although they showed no statistically significant differences (Figure 6b).

Furthermore, saturated fatty acids and unsaturated fatty acids in free fatty acids were separately analyzed. The proportion of unsaturated fatty acids significantly increased as the pores became conspicuous. On the other hand, no difference was found in the ratio of the saturated fatty acid amount (Figure. 6c).

(a)


Total sebum amount
Total sebum amount (μg)
200
150
100
50
0
*
A
B
C
volunteer groups graded by the conspicuity of the pores

(b)


Ratio of sebum components
ratio of sebum components (w/w%)
60
50
40
30
20
10
0
fatty acid
squalene
cholesterol
wax
triglyceride
Group A
Group B
Group C

(c)

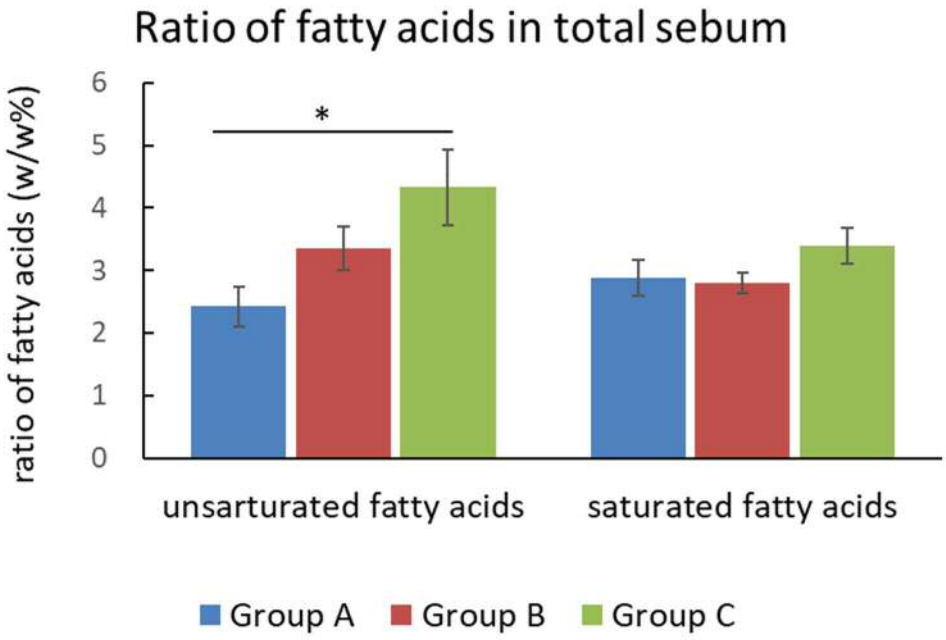


Figure 6. Sebum analysis. (a) Total sebum amount of each group graded by the conspicuity of the facial pores. ± S.E. A: n=16 B: n=23 C: n=20. (b) Analysis of sebum components of each group. Ratio of each sebum component to total sebum amount is shown. ± S.E. A: n=11 B: n=22 C: n=18 (c) ratio of the amount of unsaturated fatty acid and saturated fatty acid to total sebum amount. ± S.E. A: n=11 B: n=22 C: n=18.

1.4.2. Relationship between conspicuous facial pores and the condition of the stratum corneum

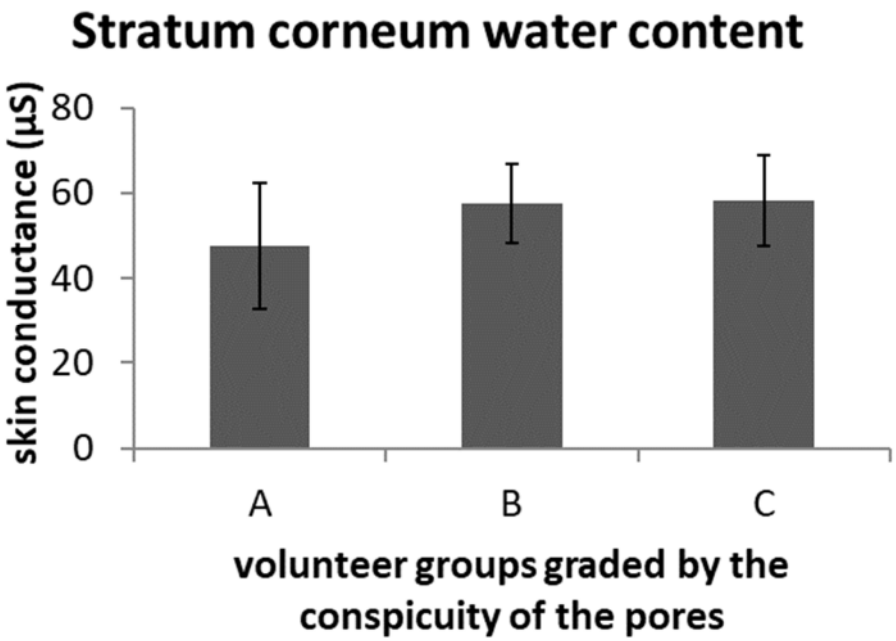
Next, the relation of the degree of conspicuity of the facial pores and the condition of the stratum corneum was investigated. Water content in the stratum corneum was measured using Skicon-200. This equipment measures the electrical conductance of the stratum corneum, which indicates its water contents. This is due to the fact that a substance which contains a large amount of water has a proportionately larger electric conductivity. The results showed that the "not conspicuous" group (A) tends to show a slightly smaller amount of water (not significant. $p>0.05$), (Figure 7a). This suggests that sebum has, to some extent, a moisturizing effect on the skin.

Next, TEWL value, an index of skin barrier function, was compared with the degree of conspicuity of the facial pores (Figure 7b). When the skin barrier function is poor, more moisture permeates through the skin. This means that the higher the TEWL value, the lower the barrier function is. A comparison between the degree of conspicuity of the facial pores and TEWL values was made. The normal pore size group (B) showed the lowest TEWL value, indicating that they have the best skin barrier function. The "not

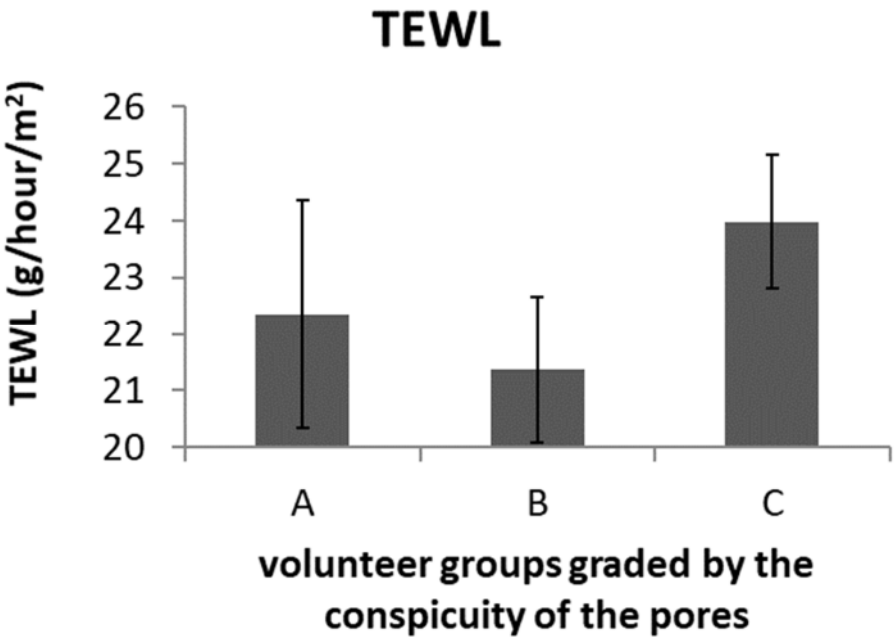
conspicuous" group (A) showed a higher TEWL value than the normal group (B). As the TEWL value of dry skin is usually high, this result is consistent with the result of skin conductance. On the other hand, the conspicuous group (C) also had a high TEWL value, although the amount of water was not small. These results indicate that the barrier function of the stratum corneum of group (C) is not normal, without resulting in dry skin condition.

Furthermore, the parakeratotic corneocytes were evaluated (Figure 7c). Parakeratotic corneocytes are the corneocytes which fail to lose their nucleus. The number of parakeratotic corneocytes is used as an index of abnormal differentiation of keratinocytes. Like TEWL values, the results showed that Group A and Group C had increased number of parakeratotic corneocytes than the normal group (B), although there was no statistical significance. This result confirmed that the differentiation of the keratinocytes of people with conspicuous pores was abnormal.

(a)


Stratum corneum water content
skin conductance (μS)
80
60
40
20
0
A
B
C
volunteer groups graded by the conspicuity of the pores

(b)


TEWL
TEWL (g/hour/m²)
26
25
24
23
22
21
20
A
B
C
volunteer groups graded by the conspicuity of the pores

(c)

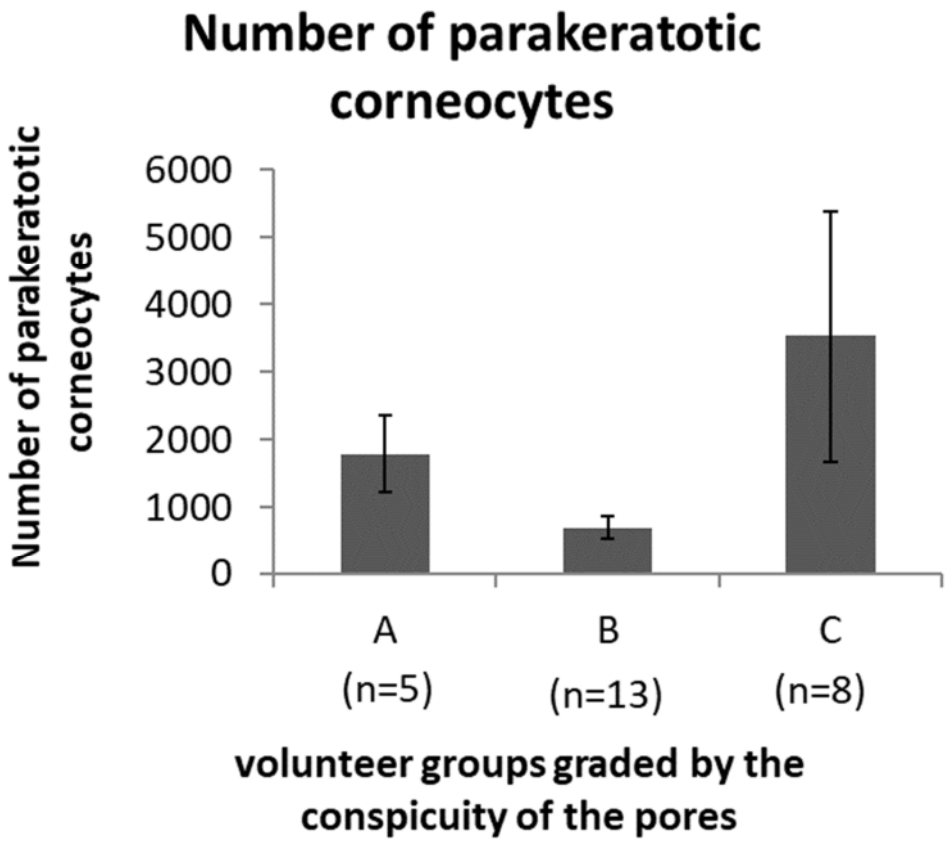
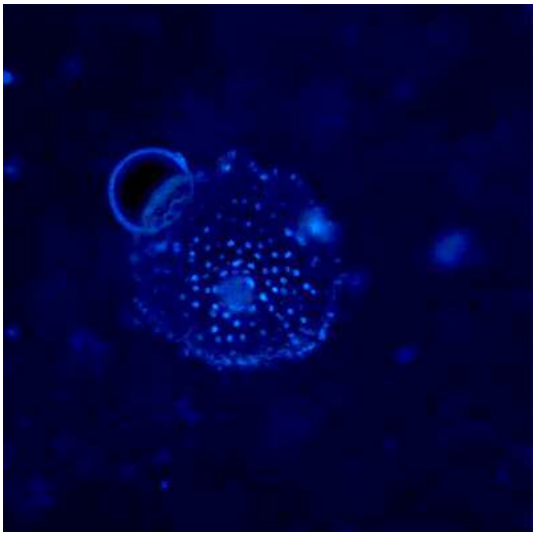


Figure 7 Parameters of stratum corneum conditions of each group graded according to conspicuity of the pores. (a) Stratum corneum water content measured by Skicon-200. (b) TEWL values measured by TEWA meter TM210. (c) Number of parakeratotic corneocytes in 780mm^2 of the stratum corneum attached to the tape.

1.4.3. Observation of parakeratotic corneocytes around facial pores

As people with conspicuous facial pores had more parakeratotic corneocytes, it was considered that parakeratosis was associated with pores. So, it was speculated that sebum secreted from facial pores damages the epidermis around the facial pores. Should this be the case, the stratum corneum around facial pores might have more parakeratotic corneocytes than that of the inter-follicular areas, which are away from conspicuous pores. Therefore, the localization of parakeratotic corneocytes was observed. Skin surface corneocytes were obtained by tape stripping. An example of an image obtained by staining a cell nucleus with Hoechst33342 is shown in Figure 8a. The number of parakeratotic corneocytes was counted using the cheeks of three healthy male volunteers who did not have a rough skin condition and the localization measurement revealed that about 95% of parakeratosis cells were around the pores of hair follicles (figure 8b).

(a)



(b)

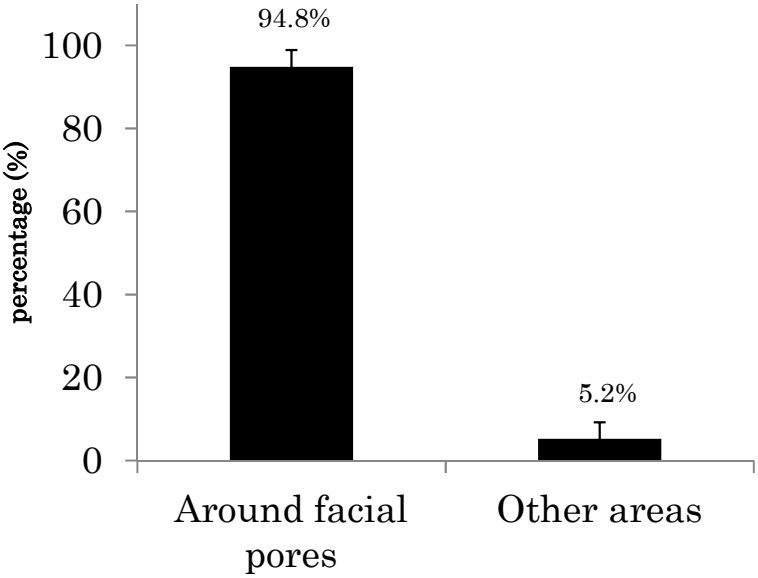


Figure 8 Localization of parakeratotic corneocytes in facial skin of normal healthy male volunteers.
(a) Representative microscopic image of parakeratotic corneocytes around a facial pore. (b) Percentage of parakeratotic corneocytes localized around facial pores.

1.4.4 Establishing a method to visualize the barrier function of the stratum corneum

Parakeratotic corneocytes were more abundant in volunteers with conspicuous pores, and most of parakeratotic corneocytes were localized around the facial pores. As TEWL was also high in ones with conspicuous pores, it was speculated that the stratum corneum barrier function around the facial pores is more deteriorated than the inter-follicular stratum corneum, which is away from the conspicuous facial pores. However, there is no method for measuring the TEWL of a small region like a facial pore. Therefore, I developed a non-invasive method to visualize the stratum corneum barrier function of a small region.

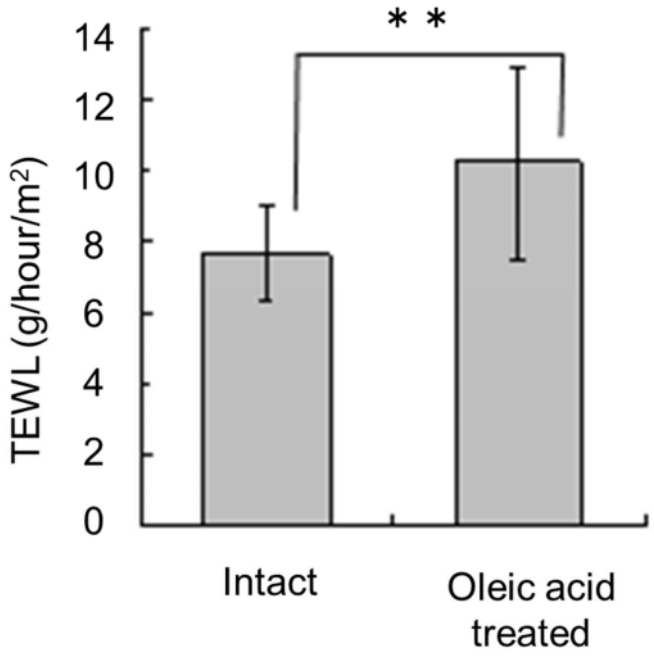
In the field of ophthalmology, the fluorescein staining method is widely used clinically as a method to detect corneal epithelial tissue damage. When the barrier function of the cornea is damaged, fluorescein penetrates and the cornea at that site is dyed. So, it was expected that this fluorescein staining method could be applied for the observation of the barrier function of the stratum corneum.

Therefore, we verified whether this method could be applied to the skin.

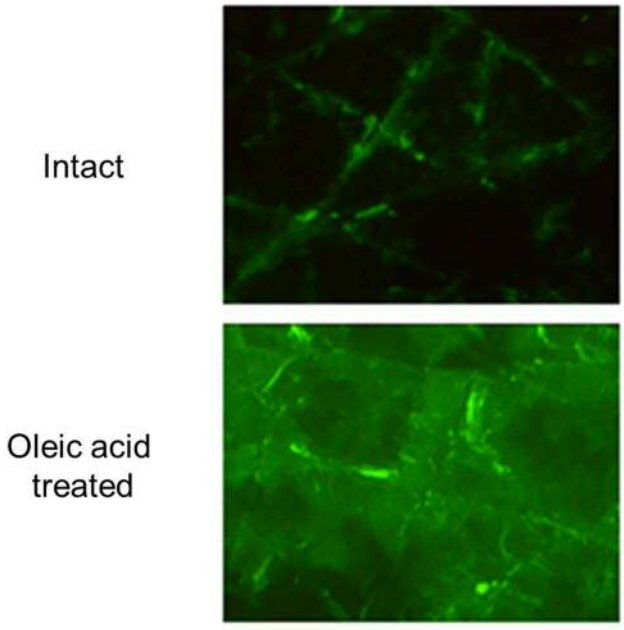
The forearm skin of a healthy male volunteer was treated with oleic acid in order to disrupt the stratum corneum barrier function. Then, fluorescein sodium was applied occlusively for five minutes. In order to observe only the penetrated fluorescein, the fluorescein that remained on the skin surface was removed by washing with water and tape stripping. After that treatment, the skin was observed with a fluorescence video microscope.

At the area of the oleic acid application, the TEWL was significantly higher, indicating that the barrier function was properly degraded (Figure 9a). Observing the absorption of fluorescein sodium at this site, more fluorescent signaling was observed compared to the intact site (Figure 9b). Fluorescence intensity of the obtained image revealed that the fluorescence intensity was significantly increased in the barrier disrupted area with the treatment with oleic acid (Figure 9c). Moreover, a significant correlation was confirmed between TEWL and fluorescence intensity (figure 9d). From these results, it was indicated that the fluorescence intensity of fluorescein reflects the barrier function of the stratum corneum. The fluorescein staining method was shown to be capable of revealing the barrier function of skin.

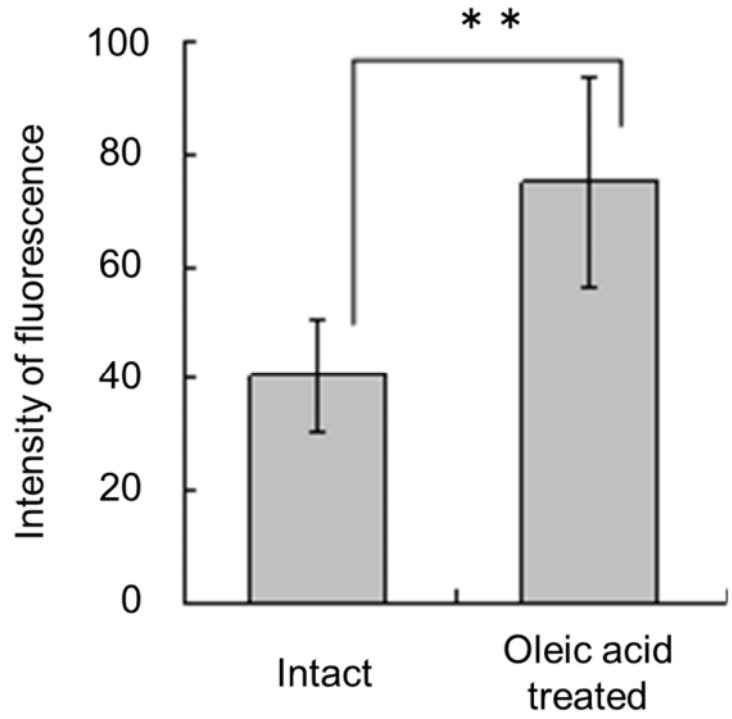
(a)


* *
14
12
10
8
6
4
2
0
TEWL (g/hour/m²)
Intact
Oleic acid treated

(b)


Intact
Oleic acid treated

(c)


* *
100
80
60
40
20
0
Intensity of fluorescence
Intact
Oleic acid treated

(d)

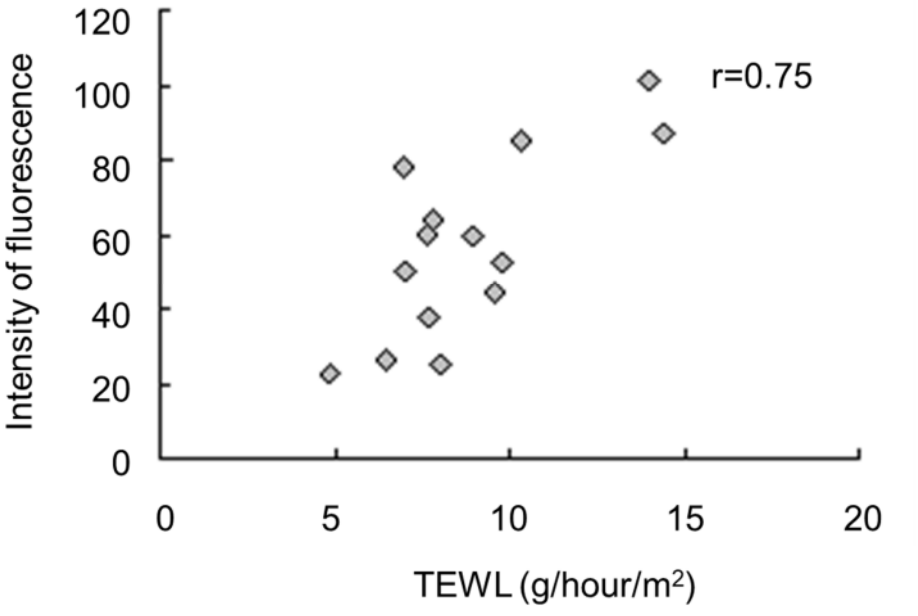

120
100
80
60
40
20
0
Intensity of fluorescence
r=0.75
0
5
10
15
20
TEWL (g/hour/m2)

Figure 9　Establishing a method to visualize the barrier function of stratum corneum.

Effects of topical application of oleic acid on barrier disruption were evaluated on forearm skin of healthy human volunteers. (a) TEWL value of the intact skin and the barrier disrupted skin with the treatment with oleic acid. (b) Representative fluorescent images of skin treated with or without oleic acid. (c) Fluorescent intensity of the images of skin treated with or without oleic acid. (d) Correlative graph of TEWL value and fluorescent intensity. ±S.D., *:$p<0.05$, ** :$p<0.01$.

1.4.5 Visualization of barrier function of the stratum corneum around facial pores

Using the fluorescein staining method established in 1.4.4, visualization of the skin barrier function around the pores in the cheek was performed. An aqueous fluorescein sodium solution was applied to the cheek skin of male volunteers for 1 minute. After washing with water and tape stripping, observation was performed with a fluorescence video microscope. As a result, fluorescent signals were observed around the pores compared with other areas (Figure. 10). This result suggests that barrier function is deteriorated around the facial pores.

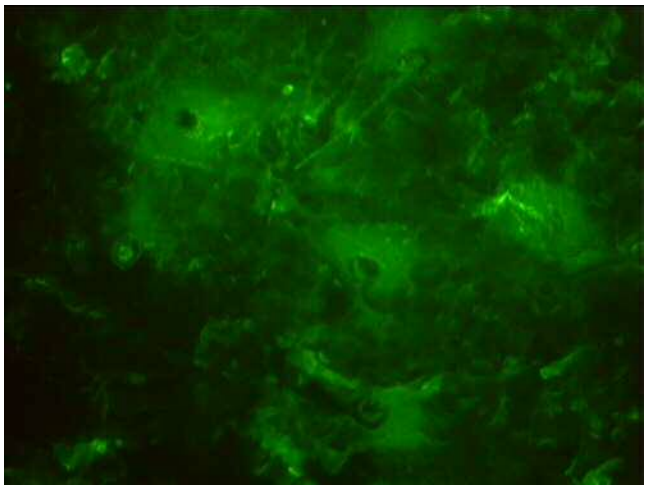


Figure 10. Visualized barrier function image of facial skin around pores.

1.5 Discussion

Sebum had been thought to have moisturizing and antibacterial behaviors to the skin. However, its details have not yet been clarified. Excessive sebum secretion is thought to have negative effects on the skin, and a typical example is acne vulgaris. In acne vulgaris, inflammation and abnormal epidermal differentiation occur in the hair follicular duct, which is the pathway of sebum secretion. The conspicuity of facial pores is also one of the adverse effects suggested to be correlated with excessive sebum. In this chapter, I tried to clarify the relationship between conspicuity of the facial pores and sebum.

First, skin measurements were conducted in female volunteers in their 20s and 30s to determine relationships between remarkable conspicuousness of facial pores and skin physiological parameters. The volunteers were classified into three groups according to the conspicuity of their facial pores. As a result of the skin measurements, as expected, the total sebum amount level was higher in the group with more conspicuous facial pores. This result supports the hypothesis that excessive sebum is one of the causes of conspicuous facial pores.

Next, the composition ratio of each sebum component was investigated. The result showed that the composition ratio of triglyceride decreased and that of free fatty acid increased as the pores became more conspicuous. This result suggests that the decomposition from triglyceride to glycerin and free fatty acids is progressing more in the group whose pores are conspicuous. There may be more skin indigenous bacteria in the conspicuous facial pores, which can decompose triglyceride.

Then, saturated fatty acids and unsaturated fatty acids in the free fatty acids were separately analyzed. The proportion of unsaturated fatty acids increased as the pores became conspicuous. Unsaturated fatty acids such as oleic acid and sapienic acid in sebum may be the candidate substrates which make the facial pores more conspicuous.

Next, the relationship between conspicuous facial pores and the condition of the stratum corneum was investigated. Water content in stratum corneum and TEWL are the most common parameters used in skin measurements. In general, when the condition of the stratum corneum gets worse, it becomes dry and its barrier function deteriorates.

Sebum is thought to have skin moisturizing effects. If sebum has no

other effects, the skin with higher contents of sebum should have better stratum corneum conditions. Indeed, our skin measurements revealed that the group with more conspicuous facial pores, which had higher contents of sebum, also had higher stratum corneum water content.

However, the conspicuous pore group with abundant sebum had a higher TEWL value, although their stratum corneum was well moisturized. It suggests that although sebum has skin moisturizing effects, excessive sebum can damage the epidermis. Numerous parakeratotic corneocytes in the group with conspicuous pores supports this idea. In addition, the condition of the stratum corneum around the conspicuous facial pores was deteriorated more than the inter-follicular stratum corneum away from the conspicuous facial pores.

Thus, we hypothesized that some sebum components might damage epidermal differentiation. As the group with conspicuous facial pores showed higher ratio of unsaturated fatty acids than the other groups, unsaturated fatty acids are the most possible candidates that trigger abnormal epidermal differentiation around the facial pores. The effects of sebum components on epidermal differentiation was investigated in Chapter 2.

While measuring the physiology of the stratum corneum around the facial pores, we also evaluated the stratum corneum barrier function around the pores. Although measuring TEWL is the most general method to evaluate barrier function, this method cannot be used in a microscopic area, like a facial pore. Therefore, the fluorescein staining method was performed to visualize the stratum corneum barrier function. This method successfully worked and provided an accurate evaluation of the cutaneous barrier function. Intensity of fluorescence was correlated to TEWL. This technique was applied to the cheek of the face and the barrier function around the pores was visually observed. The result confirmed that the barrier function of the stratum corneum around the pore was more deteriorated than other areas.

Unlike the hair follicles of the scalp, the hair follicles of the human face do not have developed hair shafts. However, hair follicles of the face have are accompanied with sebaceous glands. Well-developed sebaceous glands are characteristic to the pores of the face and scalp, which are different from the pores of the body. From the results of this study, it was suggested that excessive unsaturated fatty acids in the sebum may deteriorate the condition

of the stratum corneum around the facial pores, which may enlarge the cone-shaped structure around the pores.

Although an appropriate amount of sebum is thought to have a moisturizing effect on skin, excessive sebum is suggested to cause abnormal epidermal differentiation. It can be expected that controlling the abnormal epidermal differentiation caused by excessive sebum can suppress the conspicuity of the facial pores.

Chapter 2.

Effects of unsaturated fatty acids on the differentiation of the epidermis and the calcium influx into keratinocytes

Chapter 2. Effects of unsaturated fatty acids on the differentiation of the epidermis and the calcium influx into keratinocytes

2.1. Abstract

An excessive amount of sebum is thought to cause abnormal epidermal differentiation in conditions such as acne vulgaris and conspicuous facial pores. Calcium is a key factor of epidermal differentiation, and calcium influx into epidermal keratinocytes is reported to be associated with impaired skin barrier function and epidermal proliferation. So, I hypothesized that some sebum components affect calcium concentration in keratinocytes, which induces abnormal differentiation. To confirm this hypothesis, some lipid components in sebum were applied to hairless mouse skin and the effects on the skin were observed. Triglycerides (triolein), saturated fatty acids (palmitic acid, $C_{15}H_{31}COOH$ and stearic acid, $C_{17}H_{35}COOH$), and unsaturated fatty acids (palmitoleic acid, $C_{15}H_{29}COOH$ and oleic acid, $C_{17}H_{33}COOH$) were applied. Neither triolein

nor saturated fatty acids influence the skin barrier function or the skin surface morphology. On the other hand, the application of unsaturated fatty acids induces scaly skin, abnormal epidermal differentiation, and epidermal proliferation. In addition, unsaturated fatty acids but not triglycerides or saturated fatty acids on cultured human keratinocytes induce calcium influx into the cells. Moreover, the application of oleic acid on hairless mouse skin induces an abnormal calcium distribution in the epidermis *in vivo*. These results suggest that unsaturated fatty acids in sebum alter the dynamics of calcium in epidermal keratinocytes and induce abnormal epidermal differentiation.

2.2. Introduction

Acne vulgaris is a dermatitis often seen in the faces of adolescents, especially those with excessive sebum secretion. The first step of the formation of acne vulgaris is abnormal epidermal differentiation in the follicular ducts. Other than acne vulgaris, the conspicuity of the facial pores is suggested to be caused by excessive sebum and be accompanied by abnormal epidermal differentiation. It was suggested that some components in sebum might

damage the epidermis in Chapter 1. However, the mechanism involved in this abnormal epidermal differentiation caused by excessive sebum has not been clarified.

Human sebum is composed of many lipids, such as triglycerides, fatty acids, squalene, and esters. Triglycerides and free fatty acids together account for 57% of sebum [1]. Skin resident bacteria in the canals of hair follicles and on the skin surface, such as *Propionibacterium acnes*, hydrolyze triglycerides in the sebum and release free fatty acids. It has been reported that free fatty acids induce various membrane-associated effects at the cellular level. Previous studies demonstrated that the expression of keratin was abnormal in the upper hair follicles. Man, et al, demonstrated that topically applied lipids are incorporated into the metabolism of the epidermis [5]. Application of oleic acid on rabbit ears induced ultrastructural changes which is similar to the structure of comedo in humans [6] Thus, we can suppose that triglycerides or free fatty acids in excessive sebum might be the candidates that cause epidermal differentiation. As it was found that unsaturated fatty acids were abundant in the sebum of volunteers with conspicuous facial pores, we hypothesized that unsaturated fatty acids may

induce abnormal epidermal differentiation. However, the mechanism of the abnormal epidermal differentiation remains to be clarified.

Denda, et al, previously demonstrated that the activation of calcium-permeable ionotropic channels on the epidermal keratinocytes induced epidermal barrier disruption and epidermal hyperplasia. For example, topical application of agonists of P2X receptors or of an NMDA receptor agonist delayed barrier recovery and induced epidermal hyperplasia [7,8]. On the other hand, antagonists of these receptors accelerated barrier recovery after the barrier disruption by tape stripping. Calcium influx into epidermal keratinocytes delayed the recovery of barrier function after barrier disruption [9]. Application of metabotropic metabotropic receptors also induced epidermal proliferation via the calcium influx from the voltage-gated calcium channels [10]. Moreover, the increase of cAMP levels in the keratinocytes induced calcium influx into the cells, which lead to barrier abnormalities [11]. These results suggest that hormones or neurotransmitters, which affect the intracellular cAMP level, might cause the abnormal differentiation of epidermal keratinocytes. So, it was hypothesized that some lipid components of sebum might affect the

intracellular calcium level in epidermal keratinocytes and the calcium disturbance might induce abnormal epidermal differentiation.

In this chapter, we evaluated the effects of triglyceride, saturated and unsaturated free fatty acids on the skin of hairless mice and cultured human keratinocytes. These lipids are the major components of sebum and are the candidates that cause abnormal epidermal differentiation. There is a minor difference between mouse and human sebum composition. In the mouse sebum, most of the C16 monounsaturated acids are palmitoleic acid (16:1Δ9), and 16:1Δ7 [2]. These acids, however, are rare in human sebum. Instead, sapienic acid, (16:1Δ6), and 16:1Δ8 are the major C16 monounsaturated acids [3]. In this study, palmitoleic acid was used as C16 monounsaturated acid in every experiment because most of the experiments in this study were carried out using mice. We also used palmitoleic acid in human cell experiments in order to evaluate the effect of the same fatty acids and both *in vivo* and *in vitro* experiments.

First, we carried out the *in vivo* experiments. The sebum lipid components were topically applied on the skin of hairless mice. The morphological change of the skin surface, the barrier function, parakeratosis

and the epidermal proliferation were evaluated. Then, to understand the effects of the lipids on calcium dynamics, the effects of triglycerides and fatty acids on calcium influx into cultured human keratinocytes were investigated *in vitro*.

2.3. Materials and Methods

2.3.1. Materials

Oleic acid, palmitoleic acid, stearic acid, palmitic acid, and triolein were purchased from Wako Pure Chemical (Osaka, Japan).

2.3.2. Lipid application on the skin of mice

Aliquots of 100 μL of 10% (w/w) of each lipid in ethanol were applied on the dorsal skin of 6- to 10-week old male HR-1 mice (Hoshino Laboratory Animal Inc., Yashio, Japan) once a day, for three successive days. The number of animals subjected to each treatment was four. On the fourth day, the skin surface structure was observed using a VH-6300 digital microscope (Keyence, Osaka, Japan).

2.3.3. Detection of parakeratotic corneocytes in tape-stripped stratum corneum

The skin surface stratum corneum was obtained using Carton Tape (Nichiban, Tokyo, Japan). A 10 μg per mL solution of Hoechst33342 (Sigma, St. Louis, Missouri) in PBS was applied to corneocytes attached to the tape, followed by incubation for 30 minutes at RT and washed with water. The stained nucleosomes with Hoechst33342 were observed with a fluorescence microscope. The number of parakeratotic corneocytes in the area of a 35-mm slide film were counted.

2.3.4. *In vivo* assessment of DNA synthesis

20 μL per gram body weight bromodeoxyuridine (BrdU, (Wako Pure Chemical, Osaka, Japan) 10 mM solution was injected intraperitoneally. One hour later, skin specimens were taken from the mice. After fixation with 10% formalin neutral buffer solution (Wako Pure Chemical), the specimens were embedded in paraffin and immunostained with anti-BrdU antibodies (Oxford Biotechnology, Oxfordshire, UK). Four areas on each section were selected at

random and the number of immunostained cells per 0.4 mm of epidermis was counted using an optical micrometer.

2.3.5. Barrier function of the stratum corneum of the skin of mice.

Barrier function of the stratum corneum of the mouse skin was evaluated by measurement of TEWL with a Tewameter TM210 (COURAGE+KHAZAKA, Cologne, Germany).

2.3.6. Immunohistochemistry of loricrin

Rabbit polyclonal antibody against mouse loricrin (Covance Research Products, Berkeley, California) was diluted 500:1 with blocking solution, i.e., PBS solution including 3% bovine albumin, 10% heat-inactivated goat serum and 0.4% Triton X-100. Affinity-purified biotinylated goat anti-rabbit IgG (Vector, Burlingame, California) and ABC peroxidase (Vector) were used for immunohistochemical staining, as described by Komuves et al [12].

2.3.7. Cells and cell culture

Normal human epidermal keratinocytes from neonatal foreskin were purchased from Kurabo (Osaka, Japan) as cryopreserved first-passage cells and cultured in serum-free keratinocyte growth medium Humedia-KG2 (Kurabo). Then the cells were plated on coverslips and used in the Ca^{2+} imaging assays within two days after seeding and at less than 50% confluency.

2.3.8. Ca^{2+} imaging in single keratinocytes

Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in single keratinocytes were measured by the fura-2 method [13] with minor modifications. The culture medium of cells grown on a coverslip was replaced with balanced salt solution (BSS) of the following composition (mM): NaCl 150, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1.2, N-2-hydroxyethylpiperadine-N′-2-ethanesulfonic acid (HEPES) 25, and D-glucose 3 (pH 7.4). The cells were loaded with fura-2 by incubation with 5 μM fura-2 acetoxymethyl ester (fura2-AM) (Molecular Probes, Eugene, Oregon) at 37°C for 30 minutes, and then washed with BSS and further incubated for 30 minutes at RT to allow deesterification of the loaded

indicator to fura2. Measurements were carried out at RT using an inverted fluorescence microscope (IX70, Olympus, Tokyo, Japan) equipped with a 150 W xenon lamp and band-pass filters of 340 and 380 nm wavelengths. Image data, recorded using a high-sensitivity cooled digital CCD camera (C4742-95-12ER, Hamamatsu Photonics, Hamamatsu, Japan), were analyzed using a Ca^{2+}-analyzing system (Aquacosmos/Ratio, Hamamatsu Photonics Hamamatsu, Shizuoka, Japan).

2.3.9. Visual imaging of Ca^{2+} distribution in the epidermis

Ca^{2+} distribution imaging in the epidermis was visualized using a method reported previously by Denda et al, [14]. An 2% agarose gel membrane containing 10 μg per mL Calcium Green 1 (Molecular Probes) was formed on the slide glass, and a 10 μm in thickness frozen section was placed on the gel membrane. Images were taken using a fluorescence microscope within two hours.

2.3.10. Statistics

The results are expressed as the mean ± SD. The statistical significance of differences between 2 groups was determined by applying a two-tailed Student's t test. In the case of more than 2 groups, the significance of differences was determined by means of Tukey–Kramer method. *:$p<0.05$.

2.4. Results

2.4.1. Induction of scaly skin by applying sebum components on the skin of mice

It was suggested that excessive sebum might cause abnormal epidermal differentiation in Chapter 1. In order to address this hypothesis, first, sebum lipid components were applied on the dorsal skin of hairless mice. A 10% solution in ethanol was topically applied once a day and repeated on three successive days. On the fourth day, the skin surface morphology was observed visually (Figure 11). Figure 11a shows an untreated control, which is not scaly. Application of triolein (Figure 11b), palmitic acid (16:0) (Figure 11c), and stearic acid (18:0) (Figure 11d) did not induce scales on the skin surface. On the other hand, skin treated with palmitoleic acid (16:1) (Figure 11e) and oleic acid (18:1) (Figure 11f) showed obvious scaling, which indicated that topical application of unsaturated fatty acids induced abnormal epidermal differentiation, which was suggested in Chapter 1.

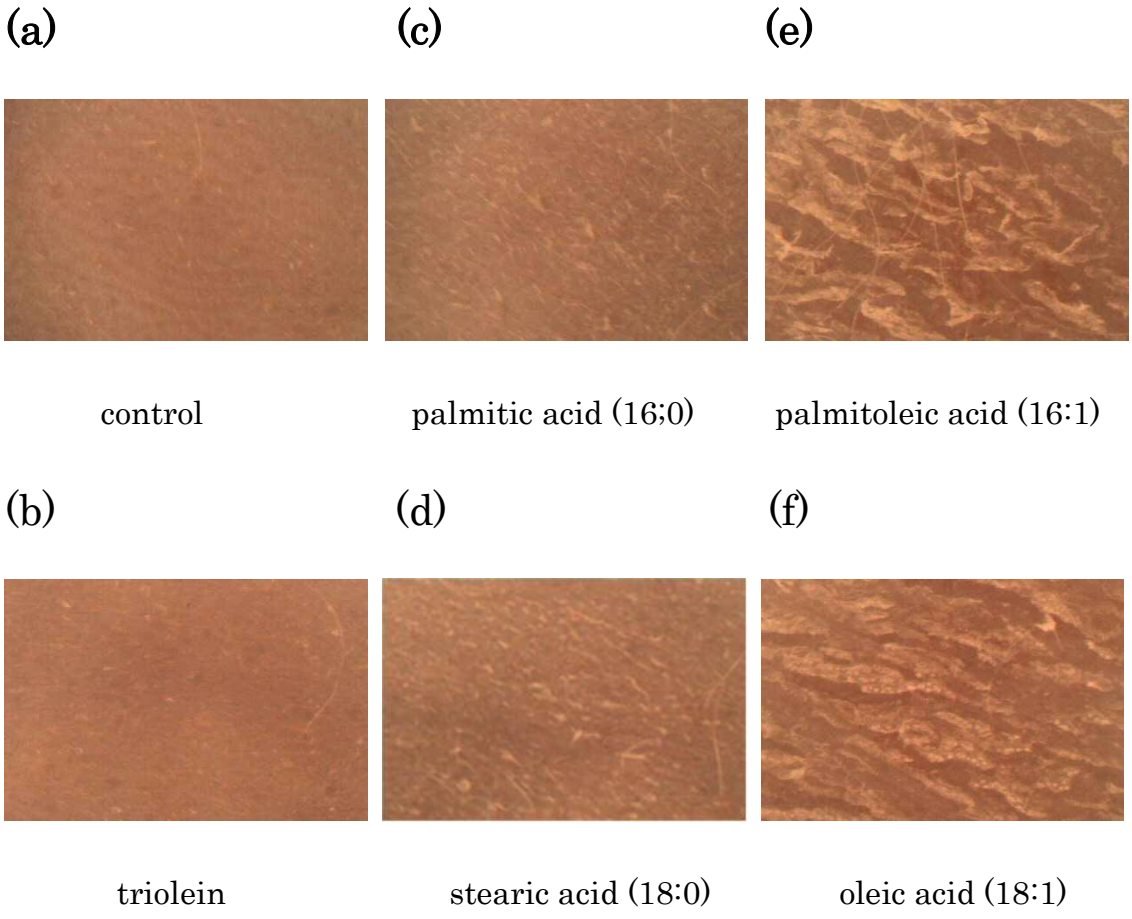


Figure 11. Skin surface morphology of mouse treated with lipids

Skin surface of untreated control mouse (a), skin surface of mouse treated with triolein (b), skin surface of mouse treated with palmitic acid (C), skin surface of mouse treated with stearic acid (d), skin surface of mouse treated with palmitoleic acid (e), and skin surface of mouse treated with oleic acid (f) are shown. Topical application of unsaturated fatty acids induced scaly skin, whereas application of triolein and saturated fatty acids did not obviously affect the skin surface morphology. Scale bar = 1mm.

2.4.2 Effects of topical lipid application on abnormal epidermal differentiation

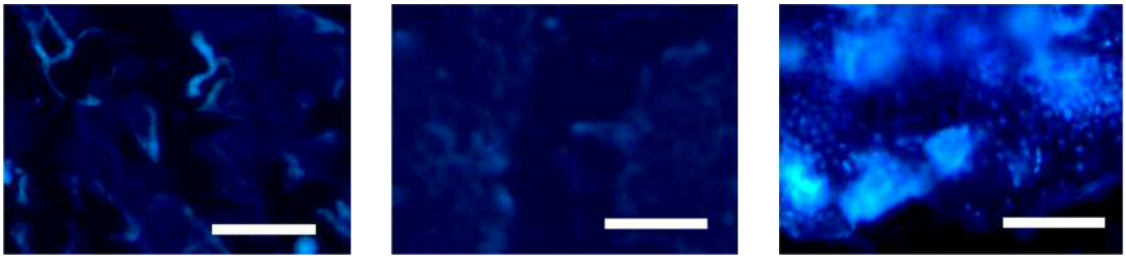
Next, the effects of the topical application of lipids on abnormal epidermal differentiation were evaluated. This abnormal epidermal differentiation was apparent in the rate of nucleus-containing cells in the stratum corneum. The outermost layer of the stratum corneum was removed by tape stripping and the nucleus-containing corneocytes in the stratum corneum were examined. In a normal epidermis, the keratinocytes lose their nuclei when they differentiate into corneocytes (Figure 12a). Application of triolein (Figure 12b), palmitic acid (Figure 12c), and stearic acid (Figure 12d) did not induce abnormal differentiation. On the other hand, application of palmitoleic acid (Figure 12e) and oleic acid (Figure 12f) did induce nucleus-containing corneocytes. We examined these parakeratotic corneocytes as an indicator of abnormal epidermal differentiation. The quantitative results are shown in Figure 12g. A significant increase in the number of parakeratotic corneocytes was detected in oleic acid- or palmitoleic acid-treated skin. Compared with unsaturated fatty acids, the damage was slight in skin treated with saturated fatty acids. Topical application of unsaturated fatty acids was

confirmed to induce abnormal epidermal differentiation.

In the case of abnormal epidermal differentiation, the localization of differentiation marker proteins must be altered. To confirm the abnormal differentiation in the mice treated with unsaturated fatty acid, we conducted immunostaining using anti-loricrin polyclonal antibody (Figure 13).

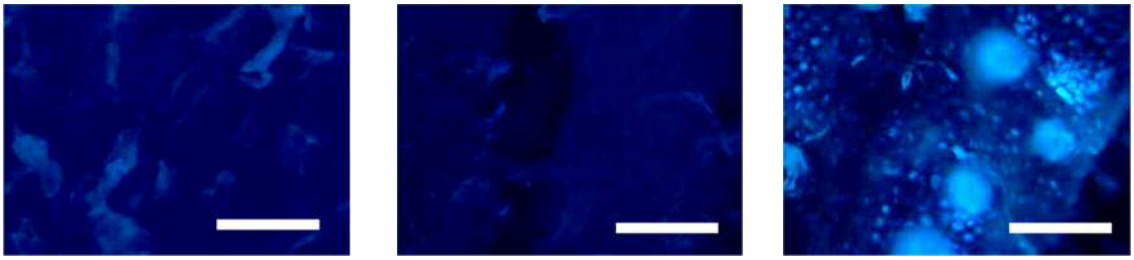
Expression of loricrin in the epidermis of skin treated with triolein (Figure 13B) was not obviously altered compared to that in untreated skin (Figure 13A). On the other hand, broader localization of loricrin was observed in the murine epidermis applied with oleic acid (Figure 13C).

(a) (c) (e)



control palmitic acid (16;0) palmitoleic acid (16:1)

(b) (d) (f)



triolein stearic acid (18:0) oleic acid (18:1)

(g)

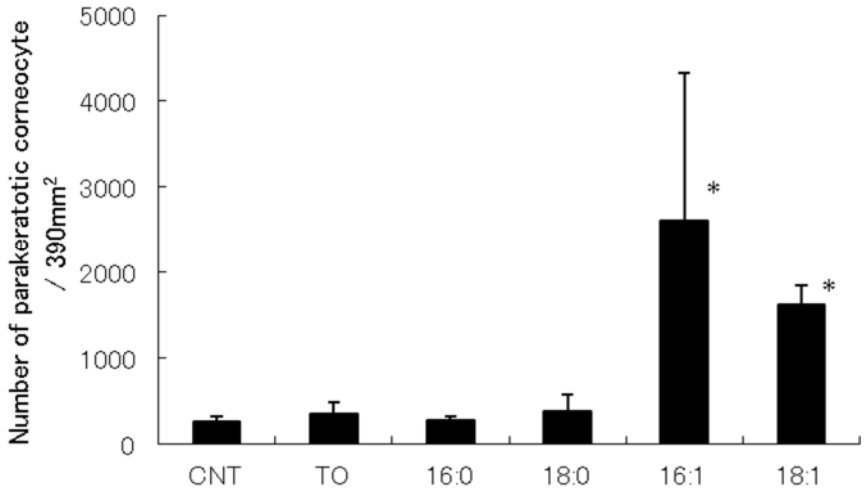


Figure 12. Parakeratosis of the epidermis after topical application of lipids

No nucleus-containing corneocyte (parakeratotic corneocyte) was observed in keratinocytes of the control (a), triolein- treated skin (b), palmitic- acid- treated skin (c), and stearic- acid- treated skin (d). On the other hand, nuclei were observed in palmitoleic- acid- treated skin (e) and oleic-acid-treated skin (f). Results of quantification in each sample are shown in (g). *:$p<0.05$, Scale bar=10 μm.

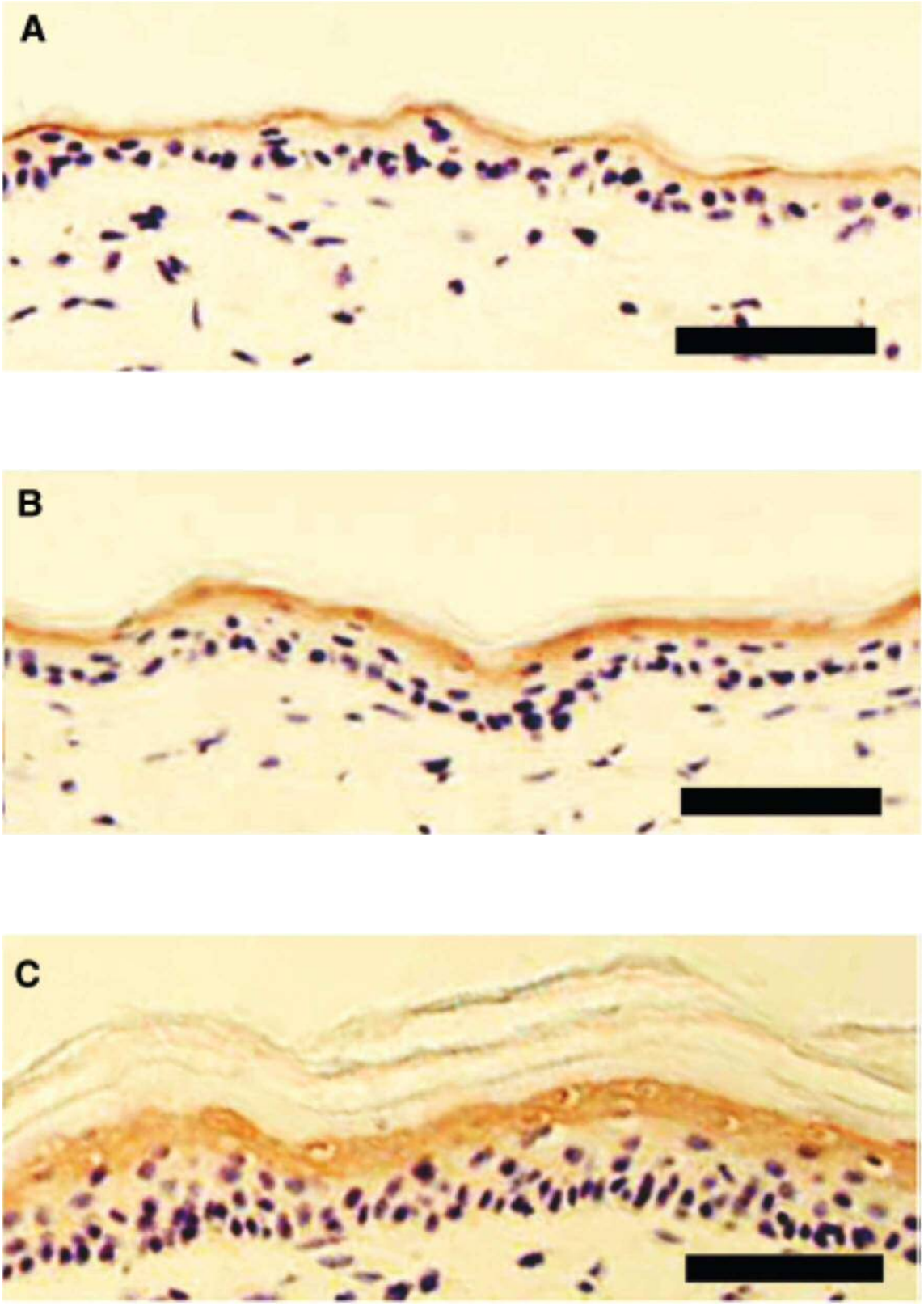

A
B
C

Figure 13. Localization of loricrin in epidermis after topical application of lipids

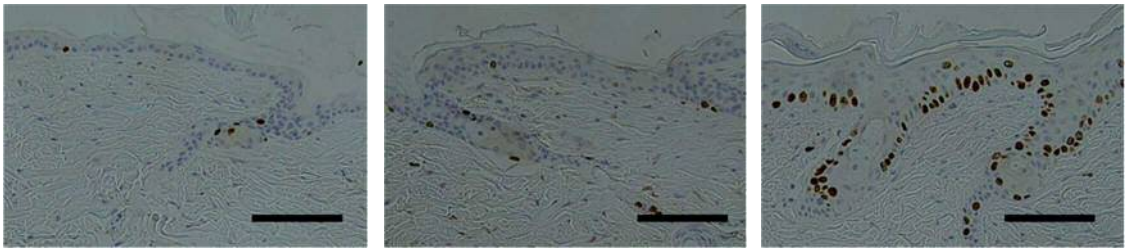
Immunoreactivity against anti-loricrin antibody was observed in the uppermost layer of the epidermis of the skin from untreated mice (A) and triolein-treated mice (B). The region which was immunoreactive to the anti-loricrin antibody was broader in the epidermis from skin treated with oleic acid (C). Scale bar=10 μm

2.4.3. Effects of topical lipid application on epidermal proliferation

Other than the effects on abnormal epidermal differentiation, we examined the effects of sebum lipids on epidermal proliferation. In order to detect proliferating cells in the epidermis, bromodeoxyuridine (BrdU) was injected intraperitoneally into mice after the three applications of lipids. BrdU is a synthetic nucleoside and is an analog of thymidine. BrdU can be incorporated into the newly synthesized DNA of replicating cells during the S phase [15].

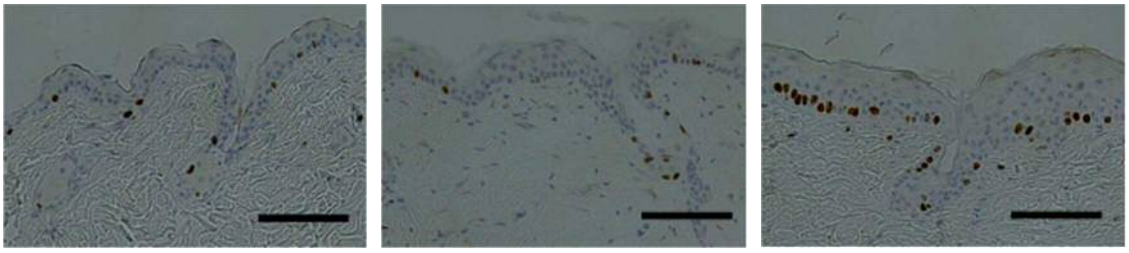
1 hour after the injection, skin specimens were obtained and were fixed and immunostained with anti-BrdU antibodies (Figure 14a– f). Untreated control skin is shown in Figure 14a. Application of triolein accelerated the proliferation slightly (Figure 14b). Application of saturated fatty acids (palmitic acid and stearic acid) did not affect epidermal proliferation (Figure 14c and 14d). Unsaturated fatty acids (palmitoleic acid and oleic acid) induced the proliferation of keratinocytes (Figure 14e and 14f). The quantified results are shown in Figure 14g. The slight increase of proliferation resulting from the application of triolein might be induced by oleic acid produced by the degradation of triolein.

(a) (c) (e)



control palmitic acid (16;0) palmitoleic acid (16:1)

(b) (d) (f)



triolein stearic acid (18:0) oleic acid (18:1)

(g)

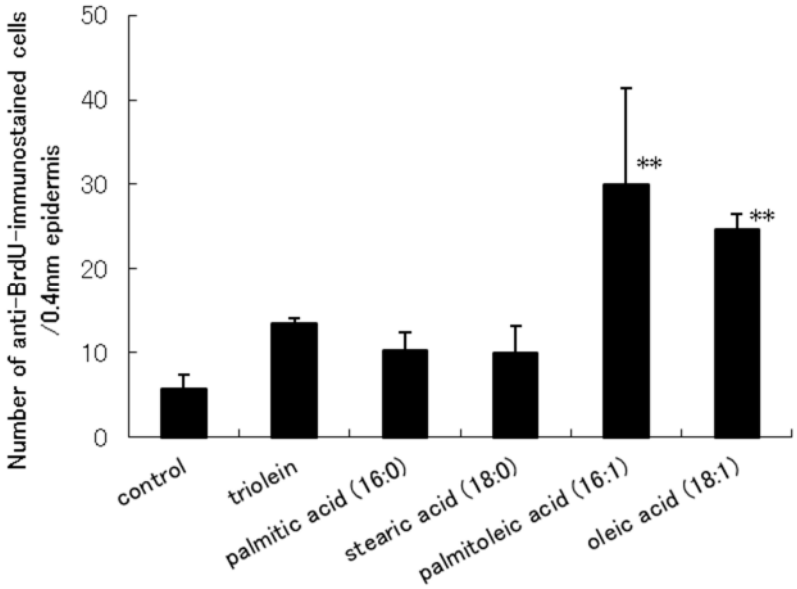


Figure 14. Epidermal proliferation after topical application of lipids

Skin section of untreated control mouse (a), skin section of mouse treated with triolein (b), skin section of mouse treated with palmitic acid (c), skin section of mouse treated with stearic acid (d), skin section of mouse treated with palmitoleic acid (e), and skin section of mouse treated with oleic acid (f) are shown. Topical application of unsaturated fatty acids accelerated the proliferation of the epidermis. Results of quantification are shown in (g). *:$p<0.05$. Scale bar=10 μm.

2.4.4. Effects of topical lipid application on barrier function

Abnormal epidermal differentiation deteriorates the structure of intercellular lipids in the stratum corneum, which causes impaired cutaneous barrier function. The effects of each lipid on cutaneous barrier function were evaluated. Compared with the control, the topical applications of unsaturated acids drastically increased TEWL. This result is consistent with the fact that unsaturated acids are well known as enhancers of trans-cutaneous drug delivery, because they disorganize the intercellular lipid lamellar structure [16].

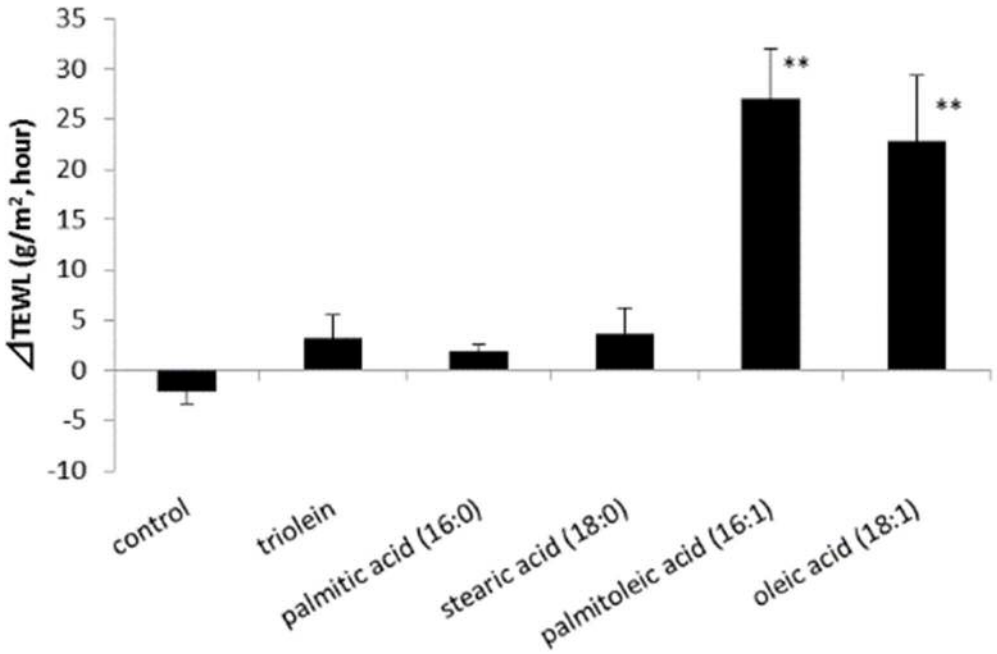


Figure 15. Cutaneous barrier function after topical application of lipids

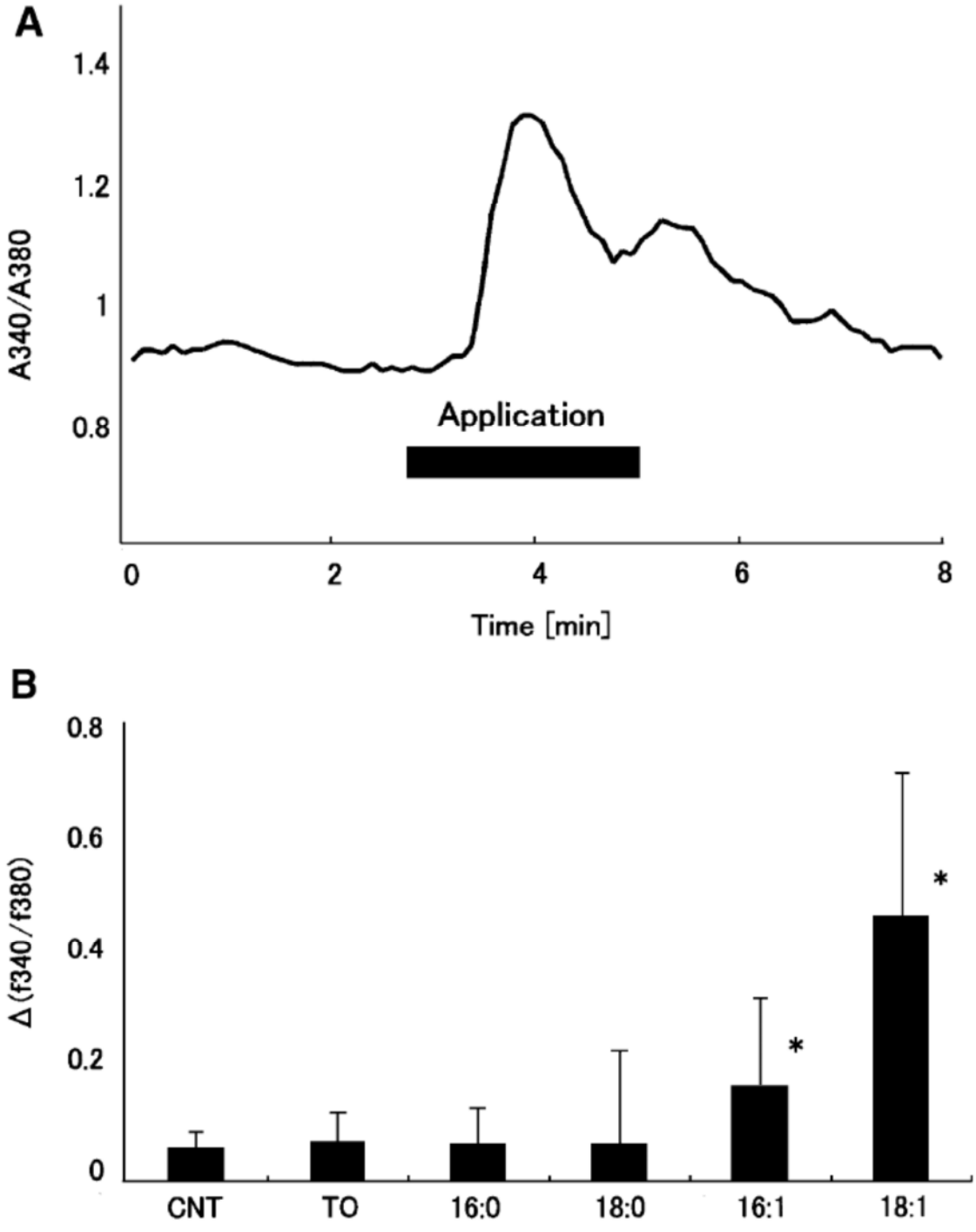
Increase of trans-epidermal water loss (TWEL) after topical application of lipids is shown. Topical application of unsaturated fatty acids drastically increased TEWL. **:p<0.01

2.4.5. Intracellular calcium concentration of cultured keratinocytes after the application of lipids

As it was demonstrated that a calcium influx into epidermal keratinocytes delayed the recovery of barrier function after barrier disruption [9], it was hypothesized that unsaturated fatty acids might affect intracellular calcium concentrations ($[Ca^{2+}]_i$). In order to verify this hypothesis, alteration of $[Ca^{2+}]_i$ of cultured keratinocytes after application of each lipid were evaluated.

We used fura-2 method to measure the intracellular calcium concentration. Fura-2 is a ratiometric and sensitive indicator dye for measuring intracellular calcium and commonly used for intracellular calcium measurements. Its structure is similar to the structure of EGTA and change its fluorescence according to calcium concentration.

Application of unsaturated fatty acids significantly increased $[Ca^{2+}]_i$ and a typical profile of this increase as induced through the application of oleic acid is shown in Figure 16a.On the other hand, saturated fatty acids and triolein did not increase $[Ca^{2+}]_i$. Quantified results of $[Ca^{2+}]_i$ alteration by sebum lipids are indicated in Figure 16b.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0.8
A340/A380
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0.4
0.2
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Δ (f340/f380)
*
*
CNT
TO
16:0
18:0
16:1
18:1

Figure 16. Intracellular calcium concentration of cultured keratinocytes after the application of lipids
(A) A representative profile of intracellular calcium levels after the application of oleic acid. Bar shows the time when oleic acid is applied to the cells. Intracellular calcium concentration increased after oleic acid was added. (B) The results of quantification. Application of unsaturated fatty acid increased the intracellular calcium concentration in cultured human keratinocytes *:$p<0.05$.

2.4.6. Calcium concentration after the application of oleic acid *in vivo*

As unsaturated fatty acids increased $[Ca^{2+}]_i$ *in vitro*, next we worked to detect the disturbance in the calcium distribution *in vivo*. An ethanol solution of oleic acid (30% w/w) was topically applied onto the skin of hairless mice for three successive days and then skin specimens were collected. Calcium was maintained at a high level only in the uppermost layer of the normal epidermis, as shown in Figure 17a. A wider distribution of calcium was seen in the epidermis of skin treated with oleic acid (Figure 17b), indicating that oleic acid disturbed the calcium distribution in the epidermis *in vivo.*

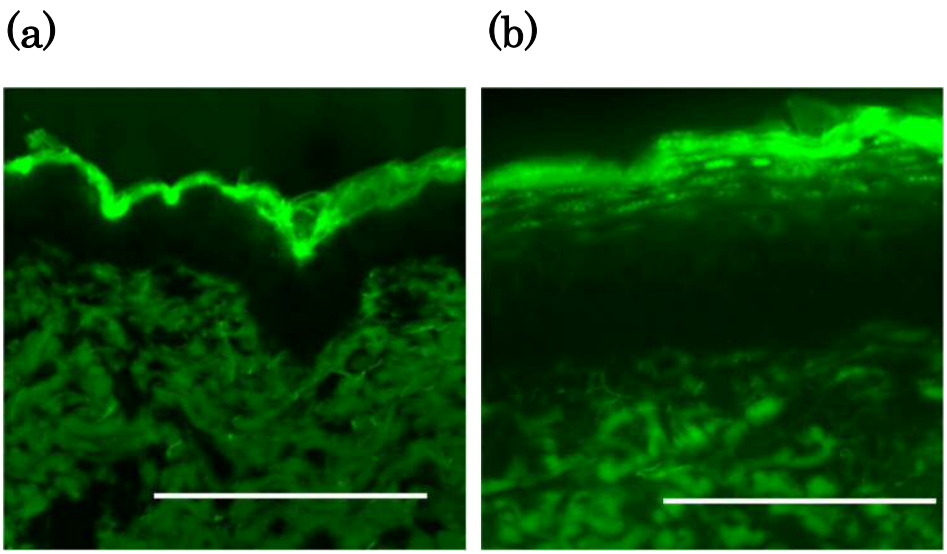


Figure 17. Visual imaging of Ca^{2+} distribution in the epidermis

(a) The calcium distribution in untreated skin. A higher concentration of calcium is observed in the uppermost layer of the epidermis. (b) The calcium distribution in the skin after the application of oleic acid. The region of higher calcium concentration is broader. Scale bar=10 μm.

2.5. Discussion

In Chapter 1, unsaturated fatty acids in sebum were suggested to be the candidates that induce abnormal differentiation. In order to address this hypothesis, sebum lipid components were applied to mouse skin *in vivo* and cultured keratinocytes *in vitro*. The results showed that unsaturated fatty acid did induce abnormal epidermal differentiation. As the calcium distribution was confirmed to be altered by unsaturated fatty acids both *in vitro* and *in vivo*, alteration of calcium dynamics may be the key factor to induce the abnormal epidermal differentiation.

In a normal epidermis, a high calcium concentration is observed only in the stratum granulosum. However, the calcium gradient was reported to dissipate immediately after loss of barrier function [14, 17], indicating that calcium distribution is important for epidermal homeostasis. The barrier function of the stratum corneum is the one of the most important roles of the epidermis. Normal epidermal differentiation and lamellar body secretion are both necessary for a healthy barrier function. The calcium gradient plays an important role in terminal differentiation of the epidermis [18] and lamellar body secretion [19].

In vivo calcium disturbance is reported in barrier disrupted disorders and ageing. Consistently, abnormal calcium distribution in the skin was reported in patients with atopic dermatitis or psoriasis [20]. Moreover, calcium gradient becomes shallower with aging [21]. Taken together, our current results suggest that changes of the calcium gradient induced by unsaturated fatty acids in sebum disturb the normal differentiation of keratinocytes.

The mechanism of the intracellular calcium influx induced by unsaturated fatty acids is still unclear. It was reported that oleic acid enhanced permeability via the stratum corneum [16]. Due to their bulky conformation, unsaturated fatty acids disorganize the intercellular lipid bilayer structure and thus reduce the barrier function. Similar to intercellular lipids, unsaturated fatty acids also affect the fluidity of the keratinocyte plasma membrane [22]. The properties of the plasma membrane may be affected by unsaturated fatty acids and this alteration may be involved in the calcium influx.

Previous studies demonstrated that mechanical stress on the epidermis induces secretion of IL-1α [23], ATP [7], and glutamate [8]. These substrates

can act as intra-cellular messengers and induce an inflammatory response or influx of calcium into the keratinocytes via various receptors. Moreover, an osmolarity-sensitive calcium channel was identified in epidermal keratinocytes [24]. Alteration of plasma cell membrane flexibility might affect the function of ion channels in the keratinocytes. In the next chapter, we evaluated the involvement of ion channel receptors in the calcium influx into keratinocytes.

The composition of unsaturated fatty acids in mouse sebum and human sebum are not the same. Palmitoleic acid (16:1Δ9) is abundant in mouse skin. However, this fatty acid is rare in humans. Instead, human sebum contains sapienic acid (16:1Δ6). Delta-6 desaturase is identified in human sebaceous glands and sapienic acid is thought to be related to the generation of acne vulgaris [25]. In this study, we used palmitoleic acid in both *in vivo* mouse experiments and cultured human keratinocytes. It had significant effects on both mouse and *in vitro* cultured human keratinocytes. More precisely, palmitoleic acid had a greater effect than oleic acid in every mouse experiment. Conversely, it had a smaller effect than oleic acid on human cells. The difference of the sensitivity to palmitoleic acid between the two species

may depend on the endogenous amount of palmitoleic acid in the sebum. Sapienic acid might have a higher effect in inducing calcium influx in human keratinocytes.

It was previously demonstrated that a decrease of environmental humidity induces abnormal skin surface morphology, scaling, and decreased desmosomal degradation [26]. Interestingly, desquamation-related enzyme activity was not altered. Unsaturated fatty acids might affect the activity of the enzymes, which regulate desquamation of the stratum corneum.

In conclusion, the topical application of unsaturated fatty acids induced the skin surface scaling, parakeratosis, and increased epidermal proliferation. Application of unsaturated fatty acids also increased the $[Ca^{2+}]_i$ in cultured keratinocytes. These results suggest that unsaturated fatty acids on the skin might increase the $[Ca^{2+}]_i$. This calcium alteration may induce the abnormal epidermal differentiation, which leads to skin troubles, such as acne vulgaris and conspicuous facial pores.

Chapter 3.

Involvement of N-methyl D-aspartate-type glutamate receptors in the function of oleic acid on the epidermal barrier and the calcium influx into epidermal keratinocytes

Chapter 3. Involvement of N-methyl D-aspartate-type glutamate receptors in the function of oleic acid on the epidermal barrier and the calcium influx into epidermal keratinocytes

3.1. Abstract

In chapter 2, it was demonstrated that unsaturated fatty acids in sebum affected calcium dynamics in epidermal keratinocytes, which may lead to a disrupted, abnormal epidermal differentiation. However, the mechanisms of these effects have not been clarified. In this chapter, in order to investigate the mechanism of the calcium influx into keratinocytes and the epidermal damage caused by unsaturated fatty acids, the involvement of calcium channel receptors was evaluated.

First, antagonists of calcium channel receptors were applied to murine skin together with oleic acid. N-methyl-d-aspartate (NMDA)-type glutamate receptor antagonists suppressed the increase of TEWL induced by oleic acid. These receptor antagonists also suppressed epidermal hyper-proliferation.

Then, the effects of these antagonists on the calcium influx into cultured keratinocytes was evaluated. NMDA receptor antagonists, MK801 and

D-AP5, inhibited the increase in the intracellular concentration of calcium ions induced by oleic acid. On the other hand, nifedipine and TNP-ATP did not inhibit the calcium influx. MK801 also suppressed the production of IL-1α.

These results suggest that the function of oleic acid on abnormal epidermal differentiation and calcium influx into keratinocytes is associated with NMDA receptors.

3.2. Introduction

Abnormal epidermal differentiation is involved in the formation of acne vulgaris and conspicuous facial pores, which are related to excessive sebum secretion. In Chapter 2, we identified unsaturated fatty acids such as oleic acid and palmitoleic acid as the casual sebum components for the abnormal epidermal differentiation and disfunction. Alteration in calcium dynamics in keratinocytes caused by unsaturated fatty acids in sebum was suggested to be related to abnormal epidermal differentiation. However, the mechanism of calcium influx caused by unsaturated fatty acid remained to be clarified.

It was reported that oleic acid enhances permeability via the stratum

corneum [16]. Unsaturated fatty acids have a lower melting point and may disorganize the intercellular lipid bilayer structure and thus reduce the barrier function. Much like intercellular lipids, it was suggested that unsaturated fatty acids affect the fluidity of the keratinocyte plasma membrane [22]. The properties of the plasma membrane may be affected by unsaturated fatty acid and this alteration may be involved in the calcium influx.

Previous studies demonstrated that mechanical stress on the epidermis induces secretion of IL-1α [23], ATP [7], and glutamate [8]. These substrates can act as intra-cellular messengers and induce an inflammatory response or an influx of calcium into the keratinocytes via various receptors.

Calcium-permeable ionotropic channels exist in epidermal keratinocytes and their activation induces barrier-related abnormalities and epidermal hyperplasia. For example, the topical application or NMDA-type glutamate receptor agonists delayed barrier repair and induced epidermal hyperplasia [8]. On the contrary, the antagonists such as MK-801 and D-AP5 accelerated the barrier recovery after the barrier disruption by tape stripping [8]. L-type voltage-dependent Ca^{2+} channel blockers, such as nifedipine, and P2X

purinergic receptor antagonists, such as TNP-ATP, also accelerated barrier recovery [27,7]. These results show that calcium channel receptors in epidermal keratinocytes are functional. These calcium channel receptors may be involved in the calcium influx induced by unsaturated fatty acids.

In this chapter, the involvement of calcium channels was investigated both *in vivo* and *in vitro* experiments. NMDA-type glutamate receptor antagonists, such as MK801 and D-AP5, specifically inhibited the calcium influx, suggesting NMDA-type glutamate receptors may be involved in abnormal differentiation induced by unsaturated fatty acids.

3.3. Materials and Methods

3.3.1. Materials

Oleic acid was purchased from Wako Pure Chemical (Osaka, Japan). MK801, D-AP5, nifedipine, and TNP-ATP were purchased from Tocris Cookson, Inc. (Ballwin, MO, USA).

3.3.2. Application of oleic acid and other compounds to the skin of mice

First, 100 µL of 10% (w/w) oleic acid in ethanol was applied on the dorsal skin of 6- to 25- week-old male hairless mouse (HR-1; Hoshino, Yashio, Japan). Then after a few minutes, 100 µL of receptor antagonist in a 30% ethanol solution was applied to the skin once a day for three successive days (n = 4). On the fifth day, TEWL values were measured with a Tewameter TM210 (Courage + Khazaka, Cologne, Germany).

3.3.3. *In vivo* assessment of DNA synthesis

A 10 mM solution of BrdU was injected intraperitoneally (20 µL per g body weight). One hour later, skin samples were taken. After fixation with a 4% paraformaldehyde solution, the specimens were embedded in paraffin and immunostained with anti-BrdU antibodies (Oxford Biotechnology, Kidlington, U.K.). In each section, the number of immunostained cells was counted and the mean value was calculated.

3.3.4. Ca^{2+} imaging in single keratinocytes

Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in single keratinocytes were measured by the fura-2 method. Normal human epidermal keratinocytes from neonatal foreskin were obtained from Kurabo (Osaka, Japan) as cryopreserved first-passage cells. The culture medium of cells grown on a coverslip was replaced with balanced salt solution (BSS) containing 150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM N-2-hydroxyethylpiperadine-N'-2-ethanesulphonic acid (HEPES) and 3 mM D-glucose (pH 7.4). The cells were loaded with fura-2 by incubation with 2 μM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR, USA.) at 37°C for 30 minutes, then washed with BSS and further incubated for 30 minutes at RT to allow de-esterification of the loaded indicator. Measurements were performed at RT using an inverted fluorescence microscope (IX70; Olympus, Tokyo, Japan) equipped with a 150 W xenon lamp and band-pass filters of 340 and 380 nm wavelengths. Image data, recorded using a high-sensitivity cooled digital CCD camera (C4742-95-12ER; Hamamatsu Photonics, Hamamatsu, Japan), was analyzed using a Ca^{2+} analyzing system (Aquacosmos/Ratio; Hamamatsu Photonics).

In the assays, the cells were preincubated with solution containing the compound (50 μM MK801, 50 μM D-AP5, 50 μM nifedipine or 100 nM TNP-ATP) for one minute and then washed away with BSS using a peristaltic pump. After two minutes, the cells were exposed to 50 μM of oleic acid for one minute.

3.3.5. Cytokine determination

Human keratinocytes (120,000 cells) were cultured in 35-mm dishes. After 48 hours, oleic acid (50 μM) and/or MK801 (50 or 250 μM) was added and after 8 hours, cells were collected for quantitative RT-PCR.

Expression levels of IL-1α, TNF-α and GAPDH were examined. Total RNA was recovered with an RNeasy Mini Kit (Qiagen, Hilden, Germany). The primers used were 5'-GTCTCTGAATCAGAAATCCTTCTA-3' and 5'-CATGTCAAATTTCACTGCTTCATC-3' for IL-1α, 5'-GGCTCCAGGCGGTGCTTGTTC-3' and 5'-AGACGGCGATGCGGCTGATG-3' for TNF-α, and 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC-3'

for GAPDH. PCR was performed as follows: initial melting (10 minutes at 95°C) and 40 cycles of amplification (15 seconds at 95°C, 5 seconds at 60°C and 17 seconds at 72°C). The expression levels were normalized to those of GAPDH as a standard.

3.3.6. Statistics

The results are expressed as mean ± S.D. Statistical significance of differences between 2 groups were determined by applying a two-tailed Student's *t*-test. In the case of more than 2 groups, the significance of differences was determined by means of Tukey–Kramer method. *$p<0.05$.

3.4. Results

3.4.1. Effects of calcium channel receptor antagonists on the increase of TEWL after the application of oleic acid

The skin damage after the topical application of oleic acid together with ion channel receptor antagonists was observed. First, 100 μL of 10% oleic acid, then 100 μL of antagonist in ethanol/water (30/70, w/w) was applied to the

dorsal skin of hairless mice. This treatment was carried out once a day and repeated on three successive days. On the fifth day, TEWL values were measured (Figure 18). As shown in Chapter 1, oleic acid induced scaly skin and caused an increase in TEWL. The NMDA-type glutamate receptor antagonists MK801 and D-AP5, each at 1 mM, specifically suppressed the increase in TEWL. On the other hand, calcium channel blockers and other receptor antagonists such as nifedipine (L-type voltage-dependent Ca2+ channel blocker, 1 mM) and TNP-ATP (P2X receptor antagonist; 10 nM) had less of an effect, although these receptors have been shown to be expressed in keratinocytes (13,14). The topical application of MK801 and D-AP5 also suppressed the proliferation of keratinocytes (Figure 19A and 19 B).

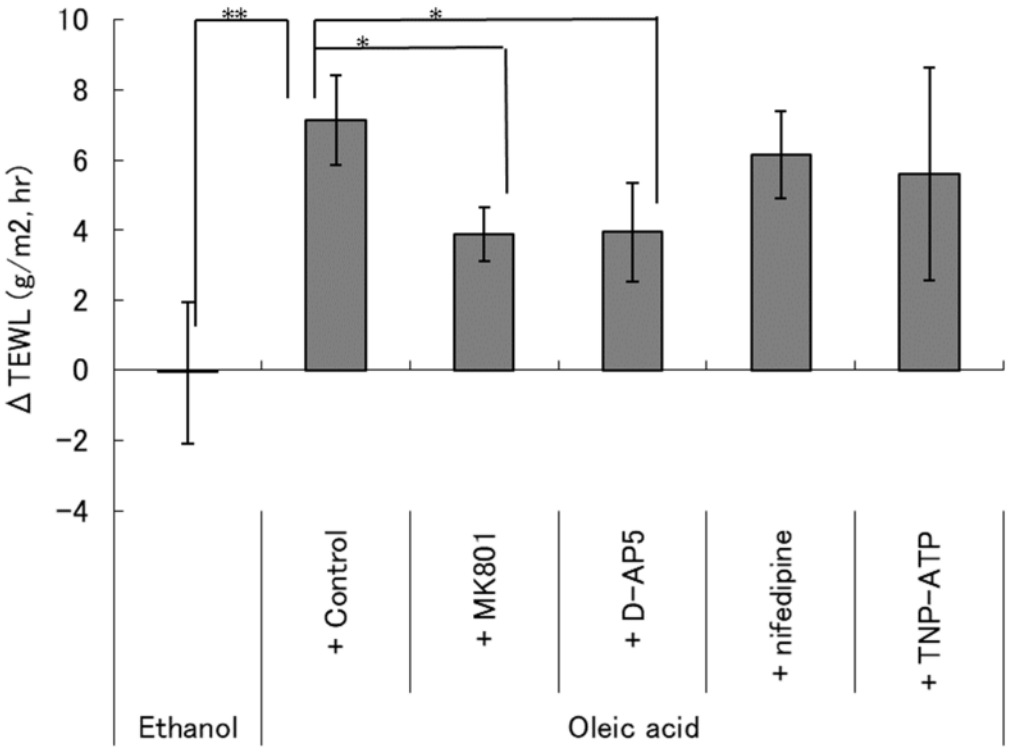
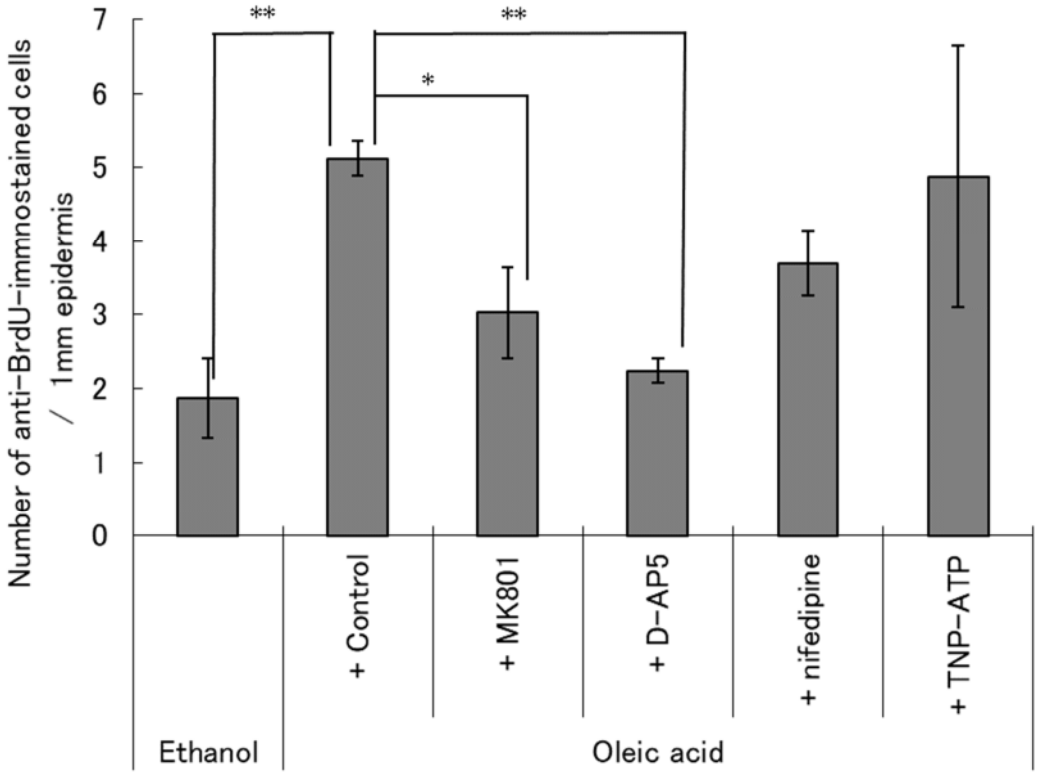
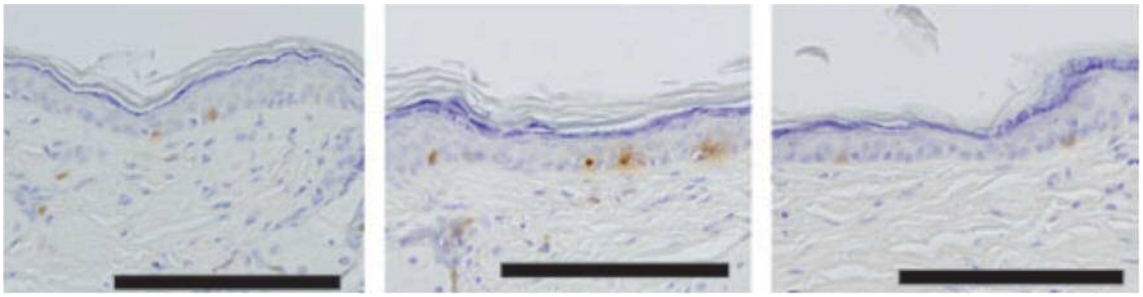


Figure 18. Effects of receptor antagonists on the increase of trans-epidermal water loss (TEWL)
Oleic acid was applied topically on the dorsal skin of hairless mouse, along with calcium channel receptor antagonists. Topical application of oleic acid increased TEWL, and MK801 and D-AP-5 suppressed the increase. *:p<0.05, **:p<0.01.

(A)



(B) (C) (D)



control oleic acid oleic acid + MK801

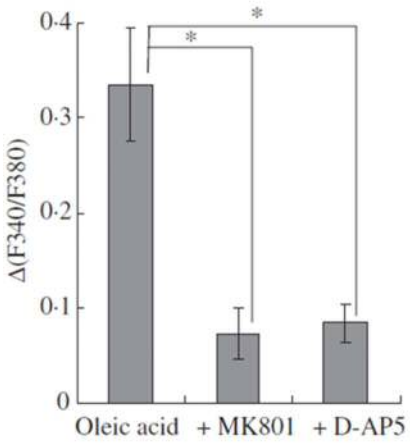
Figure 19. Effects of receptor antagonists on epidermal hyper-proliferation

(A) Number of BrdU -positive cells. Topical application of oleic acid increased the proliferation of keratinocytes *in vivo*, and MK801 and D-AP5 suppressed the hyper-proliferation. *:$p<0.05$; **:$p<0.01$. (B-D) Representative sections of BrdU staining. Control (B), oleic acid (C), and oleic acid + MK801 (D). Scale bar = 10 μm.

3.4.2. Effect of receptor antagonists on intracellular Ca^{2+} concentration in normal human epidermal keratinocytes

Next, the effects on the influx of calcium into cultured keratinocytes induced by the addition of oleic acid were examined. Addition of the NMDA receptor antagonists MK801 and D-AP5, each at 50 μM, suppressed the calcium influx (Figure 20). These results corresponded to the effects on TEWL *in vivo* and suggested that the signaling of oleic acid occurs through NMDA-type glutamate receptors.

A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Oleic acid
+ MK801
+ D-AP5

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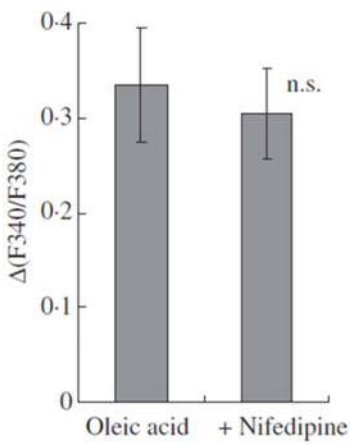
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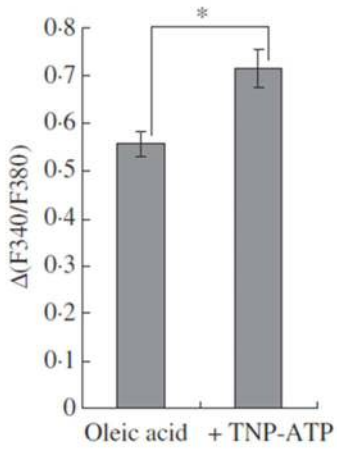
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Oleic acid
+ TNP-ATP

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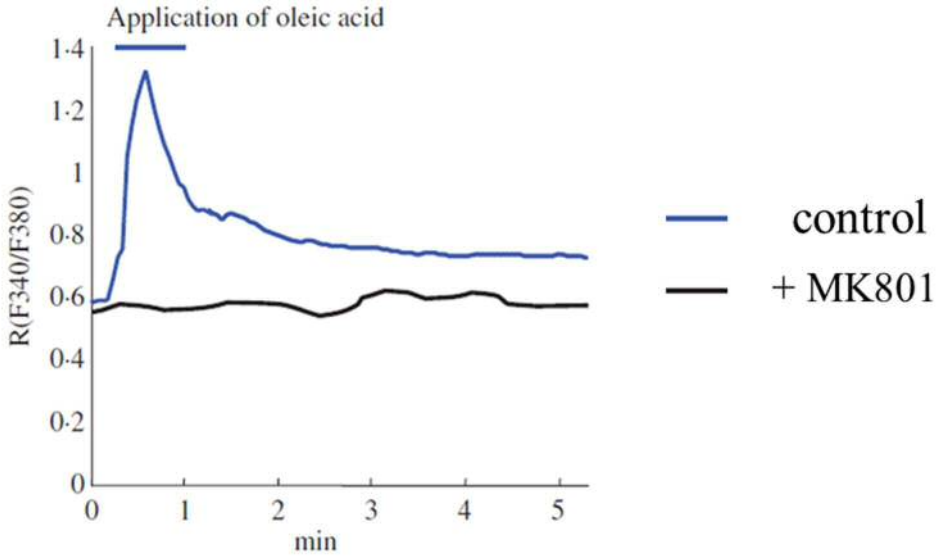
Application of oleic acid
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0
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2
3
4
5
min
control
+ MK801

Figure 20. Effects of receptor antagonists on calcium flux into keratinocytes induced by oleic acid. (A-C) The ratio of F340 and F380 is indicated as the intracellular calcium concentration. (A) Effect of MK801 and D-AP5 on the calcium flux into keratinocytes induced by oleic acid. (B) Effect of nifedipine (C) Effect of TNP-ATP. *:$p<0.05$. (D) A representative profile of the intracellular calcium level after the application of oleic acid with/without pretreatment with MK801. Bar shows the time when oleic acid is applied to the cells.

3.4.3. Effects of receptor antagonists on cytokine production

It has been reported that barrier disruption stimulates IL-1α production in the murine epidermis [23]. The effect of oleic acid on the production of IL-1α by cultured keratinocytes was estimated using RT-PCR. The addition of 50 μM oleic acid to the medium induced IL-1α production and this effect was suppressed by 50 and 250 μM MK801 (Figure 21A). The effect of MK801 on TNF-α was similar to that on IL-1α (Figure 21B). This result suggested that production of cytokines was induced by the addition of oleic acid and suppressed by the inhibition of NMDA receptors.

(A) (B)

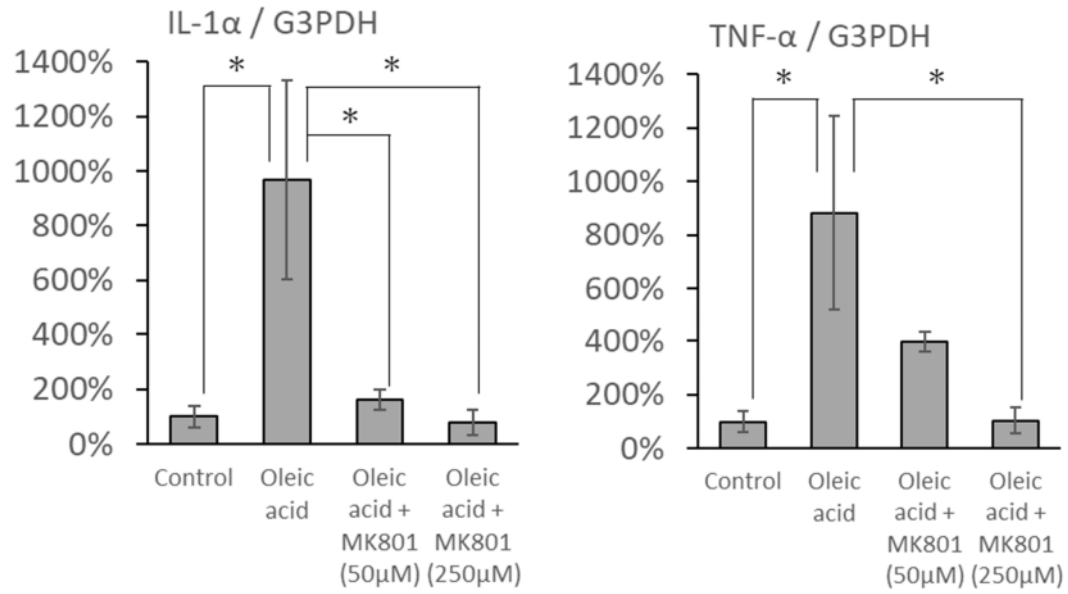

Figure 21. Expression of cytokines by keratinocytes determined using quantitative RT-PCR

(A) IL-1α and (B) TNF-α mRNA expression in control cells and oleic acid-treated cells with/without MK801. Levels are normalized to those of GAPDH. *:$p<0.05$.

3.5. Discussion

Under normal conditions, a high calcium concentration is observed only in the stratum granulosum. However, the calcium gradient dissipates immediately after barrier disruption [14,17], indicating that calcium distribution is important for the homeostasis of the epidermis.

In Chapter 2, it was shown that unsaturated fatty acids in sebum induced the calcium influx into epidermal keratinocytes and caused the abnormal epidermal differentiation. As calcium plays an important role in epidermal differentiation, the calcium disturbance caused by unsaturated fatty acids may be the trigger of the abnormal differentiation, which leads to skin problems caused by excessive sebum, such as acne vulgaris and conspicuous facial pores.

In this chapter, the mechanism of the calcium influx caused by unsaturated fatty acids was studied. We hypothesized that ion channel receptors in the plasma membrane of epidermal keratinocytes may be involved, since various kinds of ion receptors were already reported to be present in epidermal keratinocytes.

NMDA-type glutamate receptors were demonstrated to exist in the

epidermis [8,28,29] and in cultured keratinocytes [30–32]. Topical application of NMDA delayed the recovery of the epidermal barrier after tape-stripping, and MK801 and D-AP5 accelerated the barrier recovery [8]. NMDA receptors were also demonstrated to influence the $[Ca^{2+}]_i$ of keratinocytes *in vitro* [8]. These results suggest that NMDA-type glutamate receptors play an important role in epidermal homeostasis.

The result showed that antagonists of NMDA-type glutamate receptors suppress the epidermal disfunction caused by the topical application of oleic acid. These antagonists also inhibited the calcium influx caused by unsaturated fatty acid. These results suggest that NMDA-type receptors are involved in the calcium influx caused by unsaturated fatty acids.

Glutamate is a well-known neurotransmitter and NMDA-type glutamate receptors are abundant in the hippocampus in the brain [33]. The calcium influx caused by glutamate plays important role in the central nervous system. Although the function of glutamate in the epidermis is not clear, NMDA-type glutamate receptors exist and function in the epidermis. Antagonists of NMDA-type glutamate can be possible candidates for suppressing skin troubles caused by excessive sebum.

The precise mechanism of the effect of unsaturated fatty acids on NMDA receptors in the epidermis remains to be elucidated. Unsaturated (oleic, arachidonic and docosahexaenoic) fatty acids but not saturated (stearic and palmitic) fatty acids inhibit γ-aminobutyric acid-gated chloride channels [34,35]. Unsaturated fatty acids enhanced both [^{3}H]-muscimol and [^{3}H]-diazepam binding in mammals and amphibians, although the reaction was different in some fish species [35]. Arachidonic acid, docosahexaenoic acid and oleic acid are also reported to potentiate the NMDA-induced response in the pyramidal neurons of rat cerebral cortices, but palmitic acid has no effect [36]. However, oleic acid has less potential to increase NMDA-induced currents than arachidonic acid or docosahexaenonic acid [36]. In the xenopus oocyte expression system, arachidonic acid modulates NMDA receptor currents, but oleic acid does not [37]. In rat hippocampal membranes, arachidonic acid and docosahexaenonic acid interacts with ligand-binding sites of the NMDA receptor. However, oleic acid does not [38]. These reports show that some unsaturated fatty acids affect the function of NMDA-type glutamate receptors. Oleic acid may modulate the function of receptors in the epidermis, but this function may be different from

arachidonic acid or docosahexaenonic acid.

General Conclusion

Sebaceous glands are located on all parts of human skin except the palms and soles. They present in the greatest number on the face and scalp, whereas sebaceous glands on other parts of the body are far fewer. Barrier function of the facial skin is lower and skin surface texture is less organized compared to the rest of the body from the neck below. Although exposure to ultra-violet rays is regarded as the main reason for the impaired condition of facial skin, sebum may be another cause for the impaired function.

Excessive sebum is related to acne vulgaris and conspicuous facial pores. From this study, unsaturated fatty acids are shown to induce calcium influx into keratinocytes, which may be the trigger to abnormal epidermal differentiation. Furthermore, NMDA-type glutamate receptors may be involved in the calcium influx caused by unsaturated fatty acids. These findings suggest that inhibiting the function of NMDA-type glutamate receptors in the epidermis may be a new strategy for improving skin problems caused by excessive sebum.

On the other hand, the functions of sebum and unsaturated fatty acids

need further exploration and elucidation. An appropriate amount of sebum is thought to have a moisturizing effect on the skin. Indeed, skin with an insufficient amount of sebum tends to be dry skin, as shown in Chapter 1. Unsaturated fatty acids have the ability to increase the fluidity of the plasma membrane. Saturated fatty acids have less fluidity and are thought to cause arteriosclerosis. Appropriate amounts of unsaturated fatty acids may increase the fluidity of sebum, and thereby helps its secretion from sebaceous glands.

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