Unsaturated Fatty Acids Induce Calcium Influx into Keratinocytes and Cause Abnormal Differentiation of Epidermis

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Abnormal follicular keratinization is involved in comedogenesis in acne vulgaris. We recently demonstrated that calcium influx into epidermal keratinocytes is associated with impaired skin barrier function and epidermal proliferation. Based on these results, we hypothesized that sebum components affect calcium dynamics in the keratinocyte and consequently induce abnormal keratinization. To test this idea, we first observed the effects of topical application of sebum components, triglycerides (triolein), saturated fatty acids (palmitic acid and stearic acid), and unsaturated fatty acids (oleic acid and palmitoleic acid) on hairless mouse skin. Neither triglyceride nor saturated fatty acids affected the skin surface morphology or epidermal proliferation. On the other hand, application of unsaturated fatty acids, oleic acid, and palmitoleic acid induced scaly skin, abnormal keratinization, and epidermal hyperplasia. Application of triglycerides and saturated fatty acids on cultured human keratinocytes did not affect the intracellular calcium concentration ($[Ca^{2+}]_i$), whereas unsaturated fatty acids increased the $[Ca^{2+}]_i$ of the keratinocytes. Moreover, application of oleic acid on hairless mouse skin induced an abnormal calcium distribution in the epidermis. These results suggest that unsaturated fatty acids in sebum alter the calcium dynamics in epidermal keratinocytes and induce abnormal follicular keratinization.

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Abnormal follicular keratinization is involved in comedo formation in acne vulgaris, but the mechanism involved has not been clarified. Human sebum is composed of triglycerides, fatty acids, squalene, and esters, of which triglycerides and free fatty acids together amount to 57% of the total (Stewart and Downing, 1991). The free fatty acids are released from triglycerides in the sebum by bacteria in the hair canal and on the skin surface. Various membrane-associated effects at the cellular level induced by free fatty acids have been reported. Previous studies demonstrated that topically applied lipids are incorporated into the metabolism of the epidermis (Man *et al*, 1995). Hughes *et al* (1996) demonstrated abnormal keratin expression on the comedo wall and upper follicles. Application of oleic acid on rabbit ears induced ultrastructural changes similar to those in human comedo (Maeda, 1991). Thus, one can easily imagine that triglycerides or free fatty acids on the skin surface might be a cause of abnormal keratinization on the skin, although the mechanism remains to be clarified.

We recently demonstrated that the activation of calcium-permeable ionotropic channels on the epidermal keratinocytes induced barrier abnormality 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 (Denda *et al*, 2002; Fuziwara *et al*, 2003). Moreover, calcium influx into epidermal keratinocytes delayed the recovery of barrier function after barrier disruption (Denda *et al*, 2003a). Not only activation of ionotropic receptors but also activation of several metabotropic receptors induced epidermal proliferation because of the calcium influx from the voltage-gated calcium channel (Denda *et al*, 2003b). Increase of cAMP level in the keratinocytes induced calcium influx into the cell, leading to barrier abnormalities (Denda *et al*, 2004). These results suggest that hormones or neurotransmitters, which affect the intracellular cAMP level, might cause the abnormal differentiation of epidermal keratinocytes. Thus, we hypothesized that components of sebum might affect the intracellular calcium level in epidermal keratinocytes and thereby induce abnormal keratinization.

In this study, we evaluated the effects of triglyceride, saturated fatty acids, and unsaturated fatty acids on the skin of hairless mice and cultured keratinocytes. There is a difference between mouse and human sebum composition. In the mouse, most of the C16 monounsaturated acids are palmitoleic acid (16:1Δ9) and 16:1Δ7 (Wilkinson, 1970). These acids, however, are rare in human sebum. Instead, among sapienic acid, (16:1Δ6) and 16:1Δ8 are the major C16 monounsaturated acids (Nicolaides, 1974). We used palmitoleic acid as C16 monounsaturated acid in every experiment because most of the experiments in this study were carried out using mice. Moreover, we also used palmitoleic acid in human cell experiments in order to evaluate the effect of the same fatty acids and both experiments showed significant results as demonstrated later.

We first evaluated the effects of topical application lipids on the skin of hairless mice. Then, we examined the

Abbreviation: $[Ca^{2+}]_i$, intracellular calcium concentration

morphological change of the skin surface after the application of the lipids. The level of parakeratotic corneocytes and the epidermal proliferation were also evaluated. Finally, to understand the effects of the lipids on keratinocytes, we observed the effects of triglycerides and fatty acids on calcium dynamics in cultured keratinocytes and skin of hairless mice.

Results

We first observed the skin surface morphology after topical application of each lipid. A 10% solution of each lipid in ethanol was topically applied on the backs of hairless mice. This treatment was carried out once a day and repeated on 3 successive days. On the fourth day, the skin surface condition was visually observed (Fig 1). Figure 1*A* shows an untreated control. Application of triolein (Fig 1*B*), palmitic acid (16:0) (Fig 1*C*), and stearic acid (18:0) (Fig 1*D*) did not induce scales on the skin surface. On the other hand, skin treated with palmitoleic acid (16:1) (Fig 1*E*) and oleic acid (18:1) (Fig 1*F*) showed obvious scaling, which indicated abnormal epidermal differentiation.

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 normal epidermis, the keratinocytes lose their nucleus when they differentiate into corneocytes (Fig 2*A*). Application of triolein (Fig 2*B*), palmitic acid (Fig 2*C*), and stearic acid (Fig 2*D*) did not induce abnormal differentiation. On the other hand, application of palmitoleic acid (Fig 2*E*) and oleic acid (Fig 2*F*) did induce nucleus-containing corneocytes. We examined these corneocytes (parakeratotic corneocytes) as an indicator of abnormal epidermal differentiation. The quantitative results are shown in Fig 2*G* ($^*p < 0.05$). A marked increase in the number of parakeratotic corneocytes was detected in oleic acid- or palmitoleic acid-treated skin. Compared with unsaturated fatty acids, however, the damage was slight in skin treated with saturated fatty acids, whereas topical application of unsaturated fatty acids was confirmed to induce abnormal epidermal differentiation.

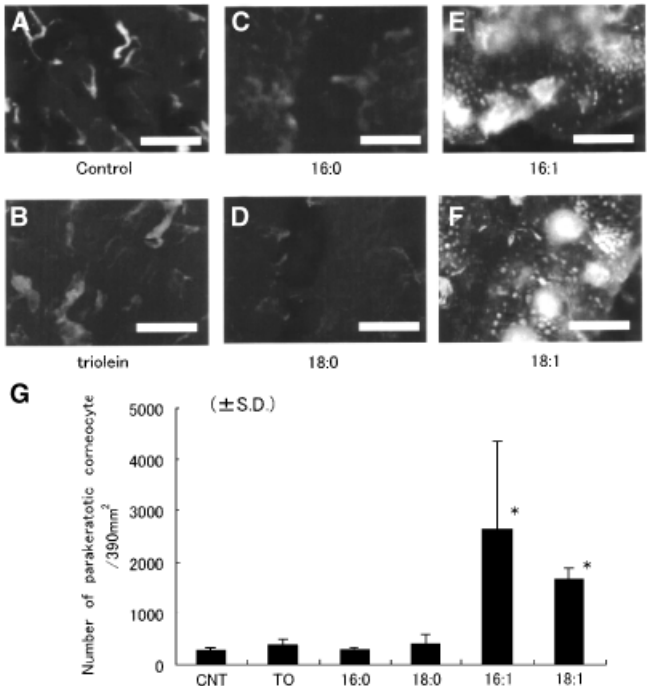

Figure 2
Parakeratosis of the epidermis was induced by topical application of unsaturated fatty acids. No nucleus was observed in keratinocytes of the control (*A*), triolein-treated skin (*B*), palmitic-acid-treated skin (*C*), and stearic-acid-treated skin (*D*). On the other hand, nuclei were observed 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.

The effects of unsaturated fatty acids on the epidermis were not restricted to abnormal differentiation. Unsaturated fatty acids also accelerated the proliferation of keratinocytes. In order to detect proliferating cells, bromodeoxyuridine (BrdU) was injected intraperitoneally into mice after three applications of lipids. The skin specimens were fixed and immunostained with anti-BrdU antibodies (Figs 3*A–F*). Untreated control skin is shown in Fig 3*A*. Application of triolein accelerated the proliferation slightly (less than 50% acceleration compared with palmitoleic acid) (Fig 3*B*). Application of saturated fatty acids did not affect epidermal

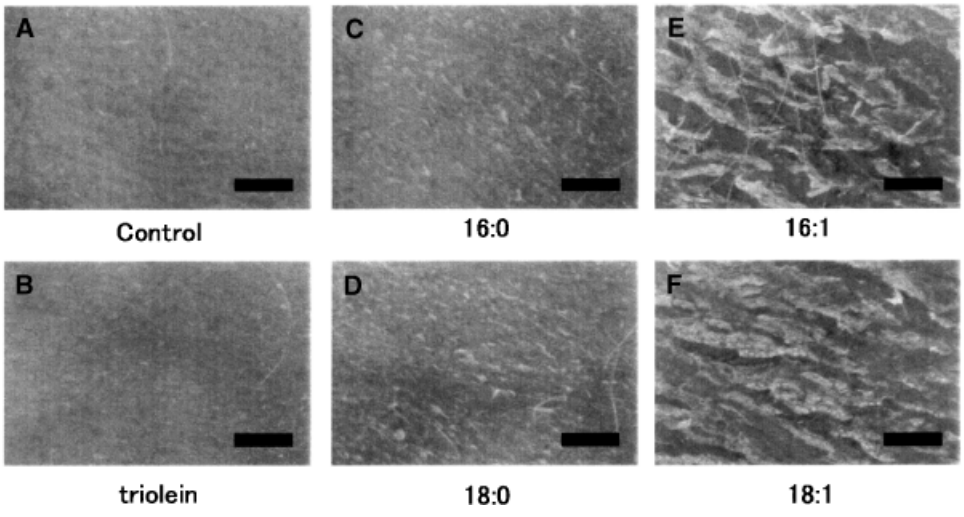

Figure 1
Topical application of unsaturated fatty acids induces scaly skin. (*A*) The untreated control is shown. Application of triolein did not affect skin surface morphology (*B*). Application of saturated acids, palmitic acid (*C*), or stearic acid (*D*) did not obviously affect the skin surface morphology. On the other hand, application of unsaturated fatty acids, palmitoleic acid (*E*), or oleic acid (*F*) induced scaly skin. *Scale bar* = 1 mm.

proliferation (Fig 3*C*, palmitic acid; Fig 3*D*, stearic acid). Unsaturated fatty acids induced proliferation of keratinocytes (Fig 3*E*, palmitoleic acid; Fig 3*F*, oleic acid). The results of quantification are shown in Fig 3*G* (*$p < 0.05$). The slight increase of proliferation by triolein might be induced by oleic acid produced by degradation of triolein.

We next evaluated the effect of each fatty acid and triolein on cutaneous barrier function. Compared with the vehicle-treated control, each agent increased the transepidermal water loss (TEWL), i.e., decreased the barrier function (Fig 4), and topical applications of unsaturated acids drastically reduced the barrier function. Unsaturated acids are well known as enhancers of trans-cutaneous drug delivery, because they disorganize the intercellular lipid lamellar structure (Potts *et al*, 1991).

In the case of abnormal differentiation, the localization of differentiation marker proteins is altered. Thus, we conducted an immunohistochemical study of treated skin samples, using anti-loricrin polyclonal antiserum (Fig 5). Expression of loricrin in the epidermis of skin treated with triolein (Fig 5*B*) was not obviously altered from that in untreated skin (Fig 5*A*). But immunoreactivity was observed over a broader area in the epidermis of skin treated with oleic acid (Fig 5*C*).

Intracellular calcium concentrations ($[Ca^{2+}]_i$) of cultured keratinocytes after application of each lipid are shown in Fig 6*B* (*$p < 0.05$). Application of unsaturated fatty acids significantly increased $[Ca^{2+}]_i$, whereas saturated fatty acids and triolein did not affect $[Ca^{2+}]_i$. A typical profile of intracellular calcium increase induced by application of oleic acid is shown in Fig 6*A*.

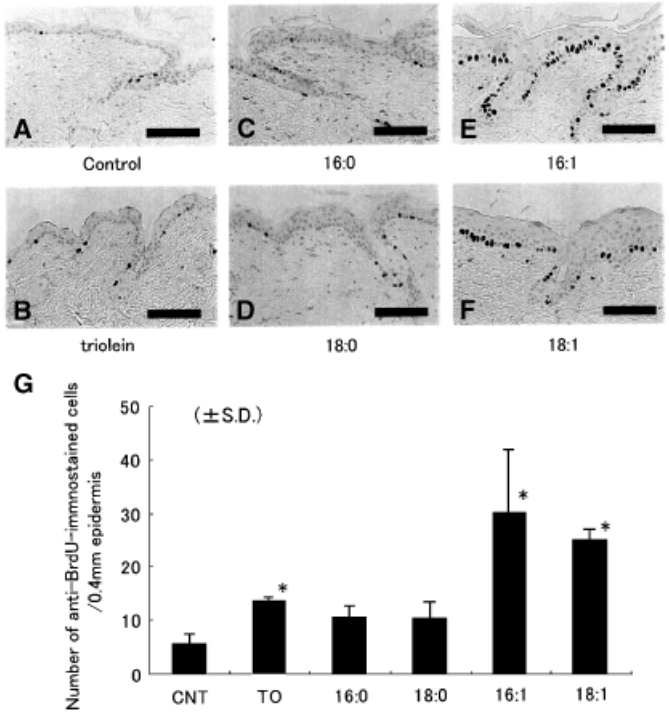

Figure 3
Topical application of unsaturated fatty acids induced epidermal hyperplasia. Representative sections are shown in (*A–F*). (*A*) Control, (*B*) triolein, (*C*) palmitic acid, (*D*) stearic acid, (*E*) palmitoleic acid, (*F*) oleic acid. Results of quantification are shown in (*G*). *$p < 0.05$. *Scale bar* = 10 μm.

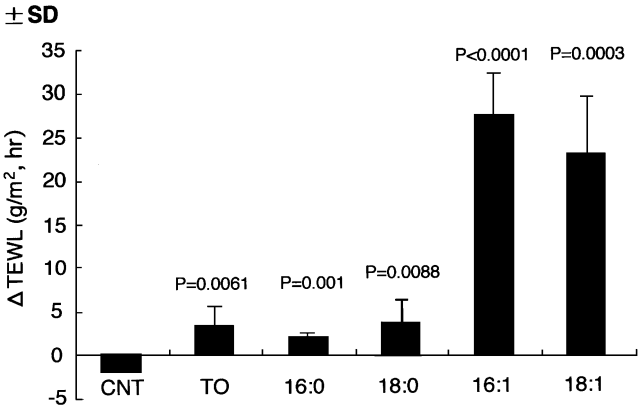

Figure 4
Topical application of unsaturated fatty acids drastically increased transepidermal water loss. Topical application of triolein and free fatty acids increased transepidermal water loss (TEWL), and unsaturated fatty acids drastically increased the TEWL.

The $[Ca^{2+}]_i$ increase in response to oleic acid was also examined *in vivo*. An ethanol solution of oleic acid (30% w/w) was topically applied onto the skin of hairless mice for 3 successive days and then skin specimens were collected. Calcium was maintained at a high level only in the uppermost layer of the normal epidermis (Fig 7*A*) whereas a wider

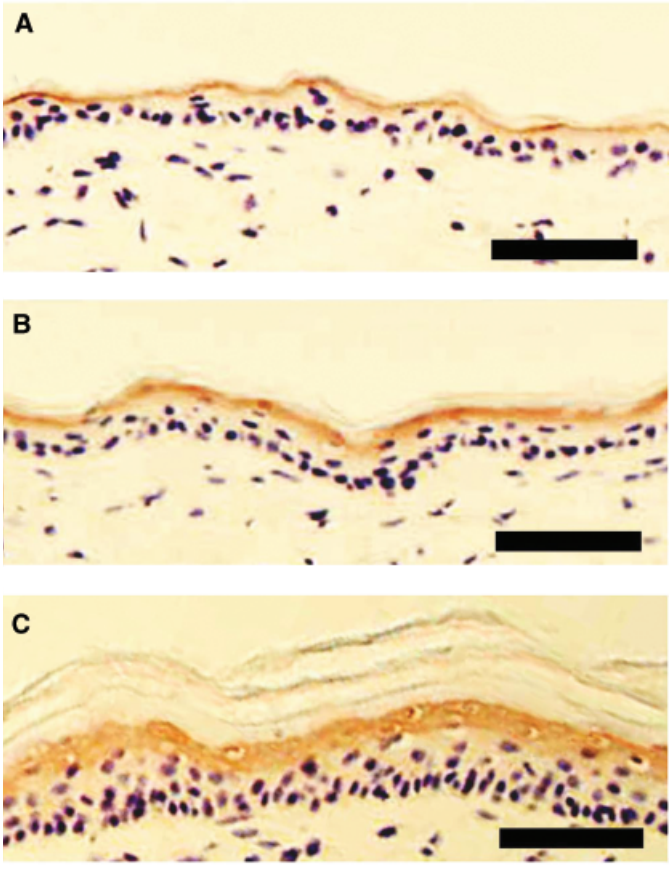

Figure 5
Topical application of oleic acid induced abnormal loricrine expression. Immunoreactivity against anti-loricrin antiserum was observed in the uppermost layer of the epidermis of the skin from untreated mice (*A*) and triolein-treated mice (*B*). The region immunoreactive to anti-loricrin antiserum was broader in the epidermis from skin treated with oleic acid (*C*). *Scale bar* = 10 μm

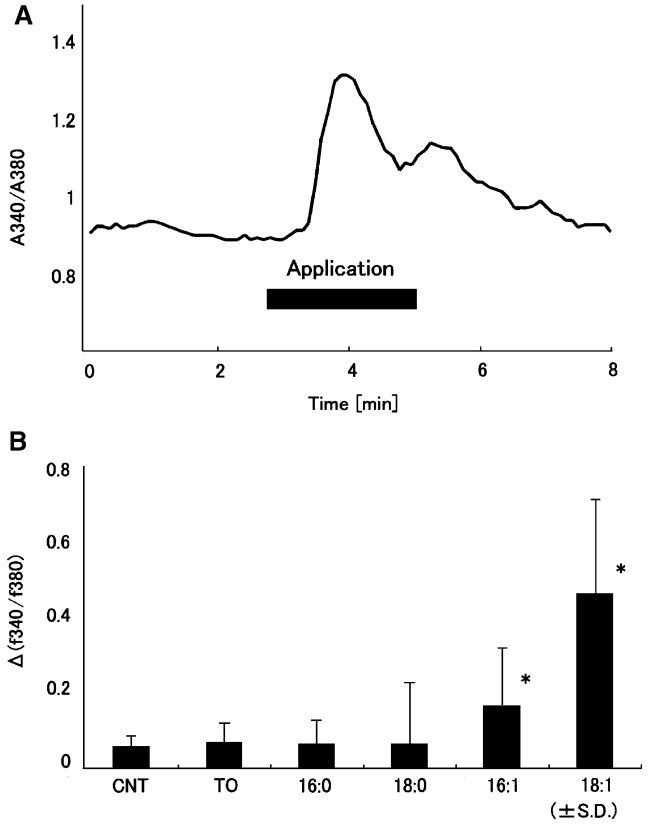

Figure 6
Application of unsaturated fatty acid increased the intracellular calcium concentration in cultured human keratinocytes. (*A*) A representative profile of intracellular calcium level after the application of oleic acid is shown. The results of quantification are shown in (*B*). $^{*}p<0.05$.

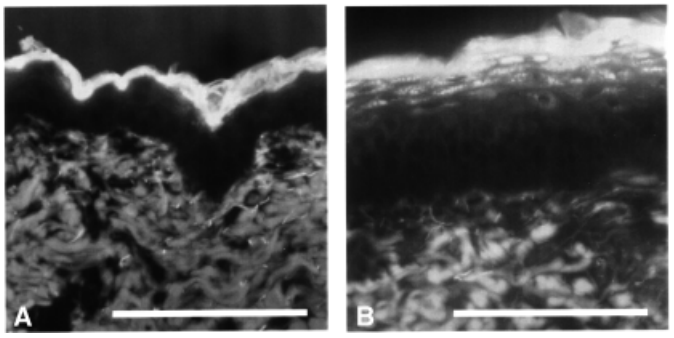

Figure 7
Topical application of oleic acid increased the calcium concentration in the uppermost layer of the epidermis. (*A*) The calcium profile of untreated skin is shown. A higher concentration of calcium is observed in the uppermost layer of the epidermis. (*B*) The calcium profile of the skin after application of oleic acid is shown. The region of higher calcium concentration becomes broader. *Scale bar* = 10 μm.

distribution of calcium was seen in the epidermis of skin treated with oleic acid (Fig 7*B*).

Thus, the $[Ca^{2+}]_i$ distribution in epidermal keratinocytes was altered by oleic acid in both *in vitro* and *in vivo* experiments.

Discussion

We recently demonstrated that an influx of calcium into epidermal keratinocytes delays recovery of the skin barrier function and induces epidermal hyperplasia (Denda *et al*, 2002; Fuziwara *et al*, 2003). In this study, the intracellular calcium distribution was altered by unsaturated fatty acids both *in vitro* and *in vivo*. In normal epidermis, a high calcium concentration is observed in the stratum granulosum, but this calcium gradient dissipates immediately after loss of barrier function (Mauro *et al*, 1998; Denda *et al*, 2000). Forslind *et al* (1999) demonstrated an abnormal distribution of calcium in skin from patients with atopic dermatitis or psoriasis. Further, the calcium gradient in normal, young epidermis becomes shallower with aging (Denda *et al*, 2003c). This calcium gradient plays an important role in the terminal differentiation of the epidermis (Elias *et al*, 2002) and in the homeostasis of the water-impermeable barrier function (Menon *et al*, 1994). Changes of the calcium gradient induced by unsaturated fatty acids might therefore interfere with the normal differentiation of keratinocytes.

The mechanism of the increase of $[Ca^{2+}]_i$ by unsaturated fatty acids is not clear. A previous study suggested that unsaturated fatty acids affect the fluidity of the keratinocyte plasma membrane (Dunham *et al*, 1996). Moreover, an osmolarity-sensitive calcium channel was identified in epidermal keratinocytes (Dascalu *et al*, 2000). Previous studies demonstrated that mechanical stress on the epidermis induces secretion of IL-1α (Wood *et al*, 1996), ATP (Denda *et al*, 2002), and glutamate (Fuziwara *et al*, 2003), which in turn might induce an inflammatory response or influx of calcium into the keratinocytes. Alteration of plasma cell membrane flexibility might well affect ion channels on the keratinocyte, but an electrophysiological study would be needed to evaluate this possibility.

Unsaturated fatty acids might be the cause of the decline of the barrier function around sebaceous glands. Oleic acid is a well-known enhancer of percutaneous drug delivery. Because of their bulky conformation, unsaturated fatty acids disorganize the intercellular lipid bilayer structure and thus reduce the barrier function. Unsaturated fatty acids also induce abnormal epidermal differentiation and proliferation, as shown in Figs 4 and 5.

Unsaturated fatty acids in mouse sebum and human sebum differ. Mouse sebum has palmitoleic acid (16:1Δ9); however, it is rare in humans. Instead, human sebum contains sapienic acid (16:1Δ6). In this study, we used palmitoleic acid in both mouse experiment and cultured human keratinocyte. It had significant effects on both mouse and human cells. But more precisely, palmitoleic acid had more effect than oleic acid in every mouse experiment (Figs 1–4). On the other hand, it had less 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 in the sebum. Sapienic acid is thought to be related to the generation of acne (Ge *et al*, 2003). In humans, sapienic acid may have a higher effect in inducing calcium influx.

We previously demonstrated that a decrease of environmental humidity induces abnormal skin surface morphology, scaling, and decreased desmosomal degradation, even though desquamation-related enzyme activity was not altered (Sato *et al*, 1998). Unsaturated fatty acids might affect the activity of the enzymes, which regulate desquamation of the stratum corneum. This should be investigated.

The face is an anatomical site in which the sebaceous gland activity is very high. The barrier function of the facial skin is lower than that of skin at other anatomical sites. Moreover, obvious comedogenesis is often observed in the facial skin. Unsaturated free fatty acids in the sebum presumably induce the abnormal condition of the facial skin. Modulation of sebum secretion or inhibition of the transformation of triglycerides to unsaturated free fatty acids might be effective to improve acne.

In conclusion, topical application of unsaturated fatty acids induced abnormal skin surface morphology, parakeratosis, and increased epidermal hyperplasia. Application of unsaturated fatty acids also increased the $[Ca^{2+}]_i$ in keratinocytes both *in vivo* and *in vitro*. These results suggest that unsaturated fatty acids on the skin might increase the $[Ca^{2+}]_i$, leading to the abnormal follicular keratinization and comedo formation that characterize acne vulgaris.

Materials and Methods

Animals All experiments were performed on 6- to 10-week-old male hairless mice (HR-1, Hoshino, Yashio, Saitama, Japan). All experiments were approved by the Animal Research Committee of the Shiseido Research Center in accordance with the National Research Council Guide (National Research Council, 1996).

Lipids application on mouse skin Aliquots of 100 μL of 10% (w/w) oleic acid (Wako Pure Chemical, Osaka, Japan) in ethanol, palmitoleic acid (Wako Pure Chemical), stearic acid (Wako Pure Chemical), palmitic acid (Wako Pure Chemical), and triolein (Wako Pure Chemical) were applied on mouse skin once a day, for 3 successive days. The number of animals subjected to each treatment was 4. On the fourth day, the skin surface structure was observed using a VH-6300 digital microscope (Keyence, Osaka, Japan).

Detection of parakeratotic corneocyte of tape-stripped stratum corneum The skin surface stratum corneum (the uppermost scaling horny layers) was obtained using Carton Tape (Nichiban, Tokyo, Japan). A 10 μg per mL solution of Hoechst33342 (Sigma, St. Louis, Missouri) in phosphate-buffered saline was applied to corneocytes attached to the tape, followed by incubation for 30 min. The stained nucleosomes were washed with water, and observed with a fluorescence microscope. The number of parakeratotic corneocytes in the area of a 35-mm slide film was counted. One sample was taken from one animal and the number of animals for each treatment was four.

***In vivo* assessment of DNA synthesis** Twenty microliters per gram body weight BrdU 10 mM solution was injected intraperitoneally (20 μL per g body weight). One hour later, mice were euthanized by inhalation of diethyl ether and skin specimens were taken. 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). On each section, four areas were selected at random; the number of immunostained cells per 0.4 mm of epidermis was counted using an optical micrometer, and the mean value was calculated. Evaluation processes were carried out in an observer-blinded fashion.

Cutaneous barrier function Permeability barrier function was evaluated by measurement of TEWL with Tewameter TM210 (COURAGE + KHAZAKA, Cologne, Germany).

Immunohistochemistry Rabbit polyclonal antiserum against mouse loricrin was purchased from Covance Research Products (Berkeley, California). The immunogen sequence was HQTQQK QAPTWPCK, which is ordinarily found at the C-terminal of the protein. The antiserum was diluted 500:1 with blocking solution, i.e., 3% bovine albumin PBS solution including 10% heat-inactivated goat serum and 0.4% Triton X-100. Affinity-purified biotinylated goat anti-rabbit IgG was purchased from Vector (Burlingame, California). ABC peroxidase (Vector) and diaminobenzidine substrate (Vector) were used for immunohistochemical staining, as described previously (Komuves *et al*, 1998). Omission of the first antibody or incubation with the substrate solution resulted in no signal, confirming the specificity of detection.

Cells and cell culture Normal human epidermal keratinocytes were obtained as cryopreserved first-passage cells from neonatal foreskin (Kurabo, Osaka, Japan). The cells were plated on coverslips, and then cultured in serum-free keratinocyte growth medium Humedia-KG2 (Kurabo). The cells were used in assays within 2 d after seeding and at less than 50% confluency.

Ca^{2+} imaging in single keratinocytes Changes in $[Ca^{2+}]_i$ in single keratinocytes were measured by the fura-2 method (Grynkiewicz *et al*, 1985) 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 min, and then washed with BSS and further incubated for 30 min at room temperature to allow deesterification of the loaded indicator. Measurements were carried out at room temperature 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).

Visual imaging of Ca^{2+} distribution in epidermis Ca^{2+} distribution imaging in epidermis was visualized using a method reported previously (Denda *et al*, 2000). An agarose gel membrane (final 2%) containing 10 μg per mL Calcium Green 1 (Molecular Probes) was formed on the slide glass, and a frozen section, 10 μm in thickness, was placed on the gel membrane. A fluorescence photomicrograph was taken within 2 h. The wavelength of the excitation light was 546 nm. For each observation, at least five sections were observed to find common features.

Statistics The results are expressed as the mean ± SD. The statistical significance of differences between two groups was determined by applying a two-tailed Student's *t* test. In the case of more than two groups, the significance of differences was determined by means of the ANOVA test with Fisher's protected least significant difference. $^*p < 0.05$.

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