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Stratum corneum lipid profile and maturation pattern of corneocytes in the outermost layer of fresh scars: the presence of immature corneocytes plays a much more important role in the barrier dysfunction than do changes in intercellular lipids

T.KUNII, T.HIRAO,* K.KIKUCHI AND H.TAGAMI

Department of Dermatology, Tohoku University School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan
**Shiseido Life Science Research Center, Yokohama, Japan*

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Summary

Background The functional characteristics of the stratum corneum (SC) of fresh scars as well as keloids and hypertrophic scars are characterized by elevated transepidermal water loss (TEWL) and increased SC hydration.

Objectives To study the composition of the intercellular lipids and maturation properties of the cornified envelope (CE) of the SC, as these are the most important components for the SC barrier function in fresh scars.

Methods SC lipids were extracted from the donor site for split-thickness skin grafting soon after re-epithelialization using a cup method, and were analysed with high-performance thin-layer chromatography. CEs, which were prepared from superficial layers of the SC, were double stained with Nile red and anti-involucrin.

Results We found a significant decrease in the proportion of ceramide (CER) in the SC lipids of fresh scars. We also observed changes in the SC CER profile that consisted of an increase in CER 4 and CER 7 and a decrease in CER 3, without any significant change in the proportion of CER 1. These changes were insufficient to explain the remarkably high TEWL recorded in the early stage of fresh scars. In contrast, with double staining of CE with Nile red and anti-involucrin, we detected the presence of numerous immature and less hydrophobic corneocytes in the outermost layer of the SC of fresh scars. Scanning electron microscopy of such corneocytes revealed numerous fine wrinkles on their enlarged surface area. Most of all, we found a closely similar, time-dependent, exponential decrease in the ratio of immature corneocytes with a poorly hydrophobic CE and in the recorded TEWL values in fresh scars. There was a highly significant positive correlation between the proportion of immature corneocytes in the outermost layer of the SC and TEWL values.

Conclusions These results suggest that the SC barrier dysfunction of the fresh scars is attributable to the presence of immature corneocytes with a less hydrophobic CE, rather than to the changes in SC lipid composition.

Key words: ceramide, cornified envelope, scar, stratum corneum

Clinically, postburn scars and surgical scars show a uniquely smooth, shiny, erythematous appearance at the early stage. Because surgical scars produced at the donor sites for split-thickness grafts show a robust zone of granulation tissue and fibrosis underlying the epidermis, it is reasonable to speculate that the homeostatic relationship normally existing between the epidermis and the dermis becomes altered, inducing changes not only in the dermis but also in the epidermis as well as in its end product, the stratum corneum (SC).[1] We previously reported similar functional

Correspondence: T.Kunii.
E-mail: kunii@mail.cc.tohoku.ac.jp

alterations demonstrable in the SC of fresh scars, hypertrophic scars and keloids.[2] These are characterized by elevated transepidermal water loss (TEWL) as well as by an increased water-holding capacity of the SC. Between these parameters the SC barrier abnormality shows more remarkable change than the water-holding capacity. In sharp contrast, the SC observed in various common types of scaly inflammatory dermatoses such as psoriasis and eczematous dermatitis demonstrates deficiencies both in water barrier function and water-holding capacity.[3] Although the unique pattern of functional changes of the SC noted in fresh scars, hypertrophic scars and keloids resembles that observed in skin treated with either systemic or topical retinoids,[4] little is known about the pathophysiology underlying the SC dysfunction in such scars. Therefore, in this study, we investigated the composition of the SC lipids and the properties of the cornified envelope (CE), both of which are considered to be major factors in the SC barrier function, as well as the morphological characteristics of the corneocytes of fresh scars.

Materials and methods

Subjects

Twenty-one Japanese patients (13 men and eight women), age range 19–86 years (mean 58), served as the test subjects. They were patients surgically treated at the Tohoku University Hospital (Sendai, Japan) for various skin diseases, and thin (0·33 mm) or thick (0·42 mm) split-thickness skin grafts, ranging from 100 to 200 cm^2 in size, were collected using the Pagett–Hood dermatome. The lower abdomen or extensor aspect of the thigh was used as the donor site of the skin graft. Examinations were conducted on the donor site for the split-thickness skin graft at 1, 3, 6 and 12 months after harvesting the grafts. We obtained SC samples as far as possible from the edge of the scars. For controls, the adjacent or contralateral normal skin of the same subject was used. This study design was approved by the medical school institutional review board. Informed consent was obtained from all the patients.

Noninvasive biophysical measurements of the stratum corneum

All the measurements were conducted in a climate chamber with the environmental conditions adjusted to a room temperature of 21 ± 1 °C and 50 ± 3% relative humidity after a 15-min acclimatization time. TEWL was measured with an Evaporimeter EP-1 (ServoMed AB, Stockholm, Sweden).[5] For the evaluation of the hydration state of the SC, we measured high-frequency conductance with a SKICON-200 (IBS Ltd, Hamamatsu, Japan). The high-frequency conductance values obtained *in vivo* are influenced only by changes of water content in the skin surface. They are little influenced by those of coexisting electroconductive components in the SC.[6] The subjects were prohibited from using any topical agents the night before.

Lipid analysis

Lipid collection. Lipid extraction was performed using a cup method with a 5-mL hexane/methanol (2 : 3) mixture for 60 s.[7] The solution was applied using a glass cylinder with a cross-sectional area of 7·5 cm^2. The cylinder was held in position manually on the extraction site for 60 s with a force sufficient to prevent lateral leakage. We chose this method because it could be carried out in a short period of time causing just tolerable pain to the subjects. Even though the lipids might be extracted mainly from the upper layers of the SC with this method, the ceramide (CER) fractions do not show any difference as compared with those obtained from the deeper layers.[8] Thereafter, solvent was evaporated at 40 °C under nitrogen. The extract was dissolved in a 0·4-mL chloroform/methanol (2 : 1) mixture and stored at −20 °C until further analysis. The amount of lipids collected showed variations between subjects. When the quantity of lipids extracted was too great, we analysed only one-third to one-half of the sample. For high-performance thin-layer chromatography (HPTLC) analysis we drew standard curves with various amounts of cholesterols (CHOLs) and CERs, confirming that the weight ratio of the SC lipid components was comparable among different samples. Therefore, we used this ratio for the purpose of comparison.

High-performance thin-layer chromatography. Thirty microlitres of the lipid extract were applied under nitrogen on the HPTLC plate (Merck, Darmstadt, Germany) using a Linomat (CAMAG, Muttenz, Switzerland) and separated by using the sequential development system described by Weerheim and Ponec[9] with some modifications: (i) dichloromethane/ethyl acetate/acetone (76 : 8 : 16); (ii) chloroform/acetone/methanol (80 : 16 : 4); (iii) hexane/chloroform/hexyl acetate/acetone/methanol (6 : 80 : 0·1 : 10 : 4). The quantification was performed by

densitometry after staining (aqueous solution containing 1·5% Cu-acetate, 5% glacial acetic acid, 1% H_2SO_4 and 1% H_3PO_4) and charring at 170 °C for 10 min. Quantification of the lipid fraction was based on the known quantities of the comigrated standards.

Evaluation of cornified envelope maturation

Preparation of cornified envelopes. We collected superficial layers of the SC by a single tape stripping, which did not cause any significant influence on the various functional parameters recorded later. The SC was obtained from the test sites by using cellophane tape (Nichiban, Tokyo, Japan) 3 × 4 cm in size. Tape-stripped SC was cut into small pieces and immersed in 1 mL of dissociation buffer consisting of 2% sodium dodecyl sulphate, 20 mmol L^{-1} dithiothreitol, 5 mmol L^{-1} ethylenediamine tetraacetic acid and 0·1 mol L^{-1} Tris–HCl buffer, pH 8·5, followed by boiling at 100 °C for 10 min. Extraction with dissociation buffer was repeated three times to remove soluble materials completely, and the remaining insoluble materials were used as the CEs.

Staining of cornified envelopes. Monoclonal antihuman involucrin (1 : 100, clone SY5; Novocastra, Newcastle upon Tyne, U.K.) was used as the primary antibody against CE components as reported previously.[10] Appropriate concentrations of the CE suspensions were dropped on to a slide glass and air-dried. They were fixed in acetone at −20 °C for 10 min and hydrated in phosphate-buffered saline. The primary antibody was allowed to react at 4 °C overnight, then fluorescein isothiocyanate-labelled antimouse immunoglobulin (1 : 100; Amersham, Little Chalfont, U.K.) was applied. After extensive washing, CEs were stained with Nile red according to the method of Greenspan *et al.*[11] with a slight modification. Briefly, Nile red (3 g mL^{-1}) solution in 75% glycerol was added to the specimen, and the fluorescence was monitored with a charged couple device camera. The proportion of the total number of CEs that was involucrin positive was used as a quantitative assessment of the occurrence of involucrin-positive CEs.

Scanning electron microscopy of the stratum corneum

The surfaces of the corneocytes obtained by tape stripping were coated with gold in a sputter-coating unit and examined with a Jeol JSM-5200 scanning electron microscope.[12]

Statistical methods

Wilcoxon signed-ranks test was used for comparisons of the corresponding groups. Factorial ANOVA with the subsequent Tukey–Kramer test was employed for comparisons of the SC lipid profiles between the groups. The correlation between the proportion of immature CEs and TEWL value was analysed by the Spearman correlation coefficient.

Results

Stratum corneum functions of scars

Re-epithelialization of the wounds at the donor sites of split-thickness skin grafts was completed at about 14 ± 2 days. However, even 1 month after harvesting the grafts there was demonstrably highly elevated TEWL, which thereafter showed a rapid decrease, being followed by a gradual decline as it approached the control level. It had almost normalized after 12 months. The resultant curve of TEWL resembled an exponential curve (Fig. 1A).

The hydration state of the skin surface, as assessed by high-frequency conductance, on the other hand, exhibited a decreasing pattern, showing an initial high level that gradually decreased to the control level (Fig. 1B). These patterns of changes were similar to those in our previous observations.[2]

Stratum corneum lipid profile of scars

A great interindividual variation was found in the quantity of the free fatty acid (FFA) fraction. As FFAs in the SC consist of those derived from the sebaceous glands and those of epidermal origin, their omission from the calculation provides more barrier-specific results about the composition of the lipids. Comparing the CER and CHOL composition, there was a significant decrease in the relative amount of CER in the SC samples obtained from the 1-month-old scars (Fig. 2A). We observed an increase in the CHOL/CER molar ratio, which was estimated to be about 1·2–1·3 : 1 in fresh scars based on the assumption that the average molecular weight of CER is about 700 and that of CHOL is 368, in contrast to about 0·9 : 1 in the normal control skin.

The CER profiles of the SC of the fresh scars are shown in Figure 2 (B). There were significant increases in the proportions of CER 4 and CER 7, in contrast to a significant decrease in CER 3 in the SC obtained from 1- to 3-month-old scars.

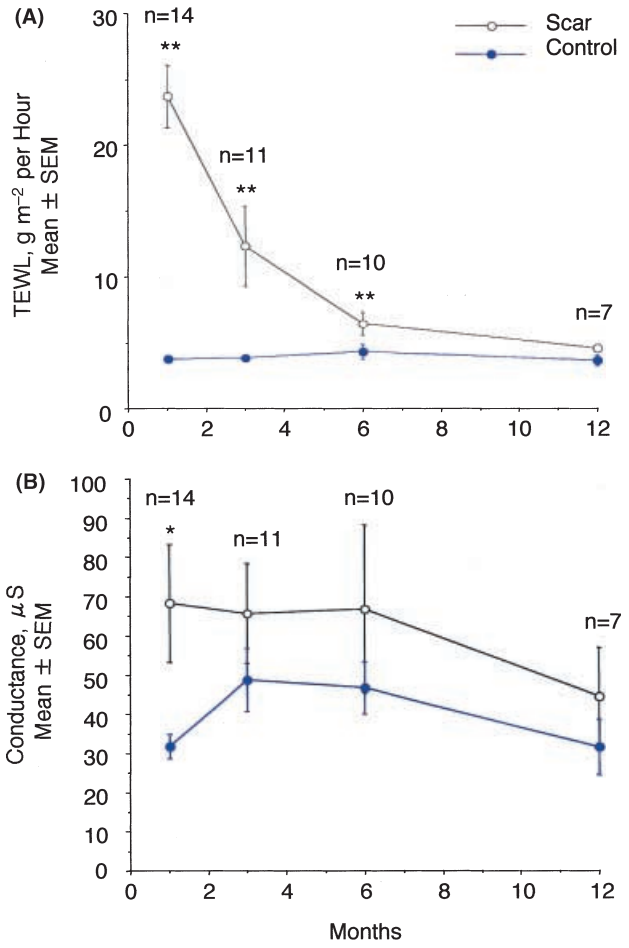

Figure 1. Changes of transepidermal water loss (TEWL) (A) and high-frequency conductance (B) measured on the scars at the donor sites for split-thickness skin grafts on the thigh or lower abdominal area (**$P < 0{\cdot}01$, *$P < 0{\cdot}05$, Wilcoxon signed-ranks test).

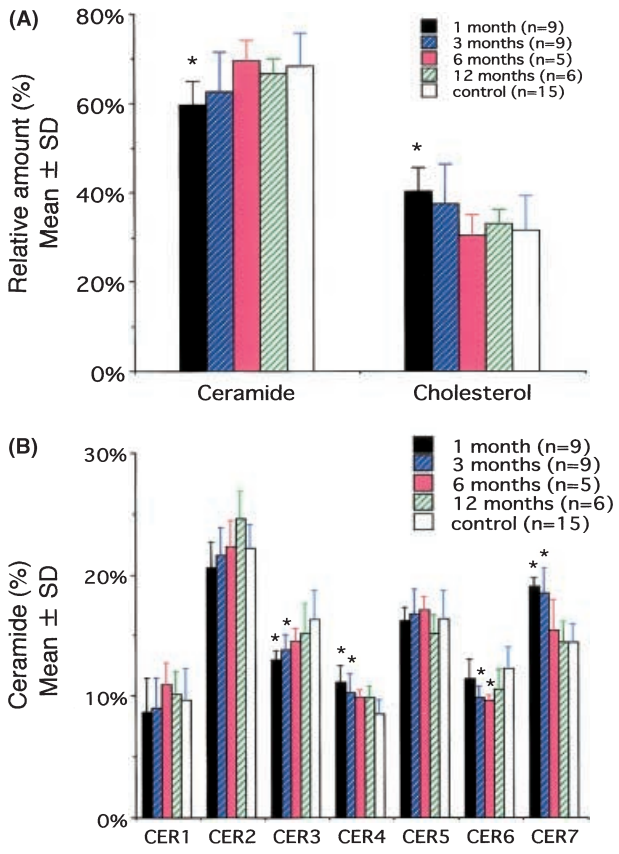

Figure 2. Changes in ceramide (CER) and cholesterol composition (A) and CER profile (B) of the stratum corneum lipids of fresh scars. There was a statistically significant decrease in the proportion of total CER at 1 month, and a relative increase in CER 4 and CER 7 and a decrease in CER 3 at 1 and 3 months (*$P < 0{\cdot}05$, factorial ANOVA with the subsequent Tukey–Kramer test).

Corneocyte maturation of fresh scars

Corneocytes obtained from the outermost SC of fresh scars, especially from the 1-month-old scars, showed a remarkably high involucrin-positive ratio as compared with those of the normal controls (Fig. 3). There was a time-dependent exponential decrease in this ratio and the resultant curve closely resembled that of TEWL. Moreover, morphologically, these corneocytes with involucrin-positive immature CEs obtained from the outermost SC of the fresh scars were characterized by their irregular shape, fragility and less hydrophobic nature as revealed by Nile red-negative reactivity (Fig. 4). As stated above, we found similar exponential curves with the TEWL values and involucrin-positive corneocyte ratio in the SC. In fact, there was a significant positive correlation between the TEWL level and the proportion of involucrin-positive CEs in the superficial portion of the SC ($n = 70$, $P < 0{\cdot}001$, $r = 0{\cdot}489$; Fig. 5).

Electron microscopic characteristics of the corneocytes in fresh scars

Scanning electron microscopic examination revealed that the corneocytes obtained from the skin surface of the fresh scars tended to be much larger and flatter than those of the normal control skin, showing numerous fine wrinkles on their surfaces (Fig. 6).

Discussion

It is generally accepted that the intercellular lipids of the SC, which are mainly composed of CERs, CHOLs and FFAs, play a crucial role in maintaining the barrier function of the SC.[13] It has been suggested that CER,

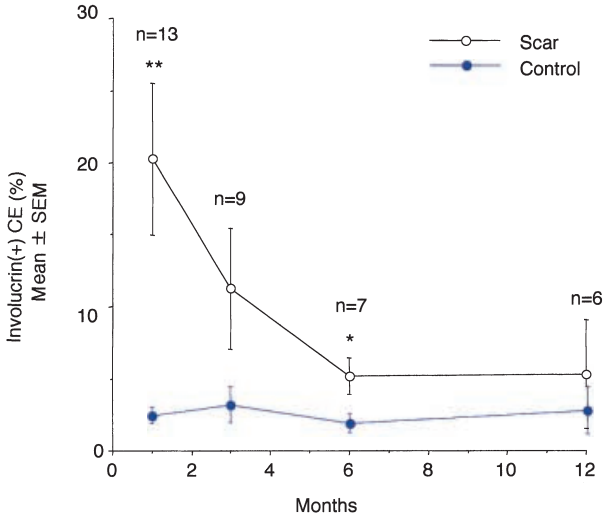

Figure 3. Changes in the frequency of involucrin-positive cornified envelope (CE) in the stratum corneum of fresh scars. Note the pattern of normalization similar to that of transepidermal water loss (**$P < 0{\cdot}01$, *$P < 0{\cdot}05$, Wilcoxon signed-ranks test).

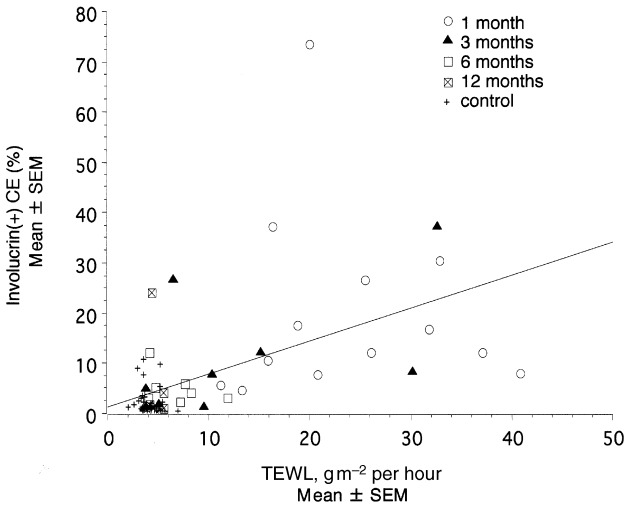

Figure 5. Relationship between transepidermal water loss (TEWL) and the proportion of involucrin-positive cornified envelopes (CEs) ($n = 70$, $P < 0{\cdot}001$, $r = 0{\cdot}489$).

CHOL and FFAs should be present at an equimolar ratio to maintain the competent barrier function of the SC.[14] Among various CER fractions, CER 1 is considered to be essential for the permeability barrier function, being thought to serve as a molecular rivet that stabilizes the multilamellar lipid array in the SC.[15] A decrease in CER 1 has been reported in several scaly inflammatory disorders such as atopic dermatitis[16–19] and psoriasis vulgaris[20] that show increased TEWL levels, and has been regarded as a cause of the skin barrier dysfunction in these disorders.

With X-ray diffraction analysis, Bouwstra *et al.* demonstrated that the phase behaviour of the CHOL/CER mixture was not sensitive to changes in the CHOL/CER molar ratio over a wide range.[21] They also reported that when CER 1 was missing there was almost no formation of a 12·8-nm lamellar phase, which is also referred to as a long periodicity phase, even at the optimal 1 : 1 CHOL/CER ratio. This phase

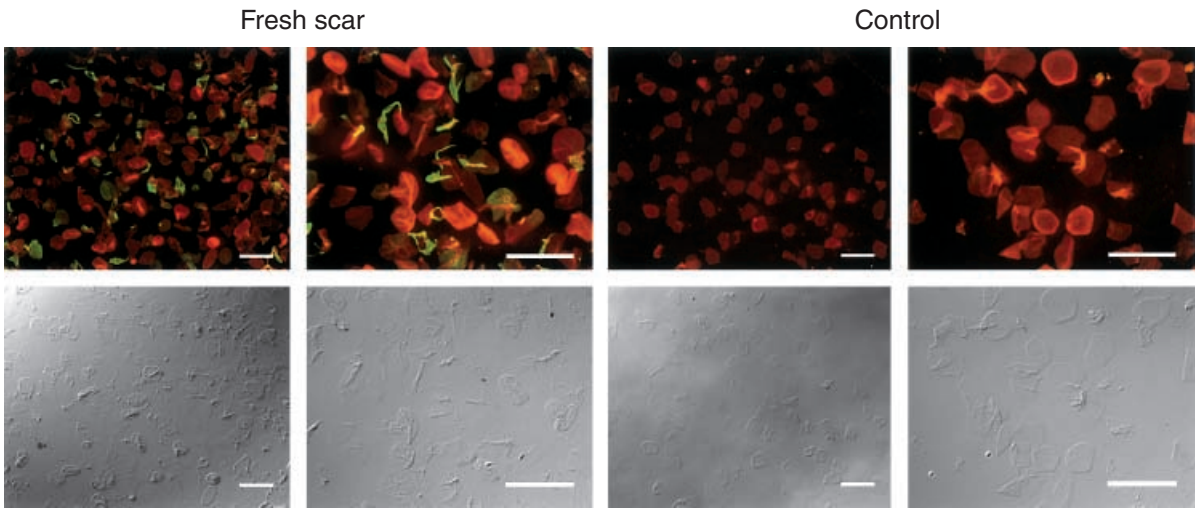

Figure 4. Double staining patterns of cornified envelopes (CEs) in corneocytes from a fresh scar and from a normal control with Nile red and anti-involucrin. CEs were prepared from the outermost stratum corneum of fresh scars and that of a normal control. Upper panel, double staining with anti-involucrin and Nile red; lower panel, phase contrast image. Scale bars = 100 μm.

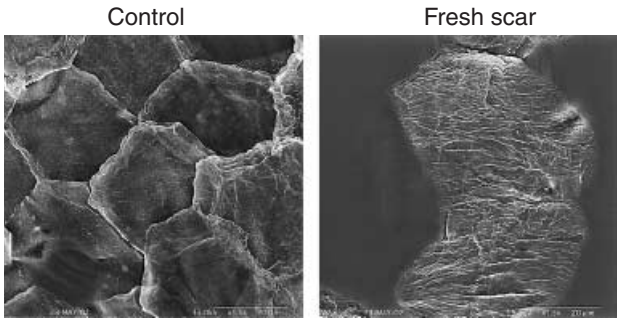

Figure 6. Scanning electron micrographs of outermost corneocytes obtained by tape stripping from normal skin and from a fresh scar. Original magnification ×1500; scale bars = 20 μm.

has been considered to be very important for the permeability barrier function of the skin, because this phase is present in all examined species, and is indicative of the unusual organization of SC lipids.[21] Moreover, except for the absence of CER 1, the phase behaviour of equimolar mixtures of CHOL and CER was rather insensitive to changes in the CER fraction.[22]

In the present study, we observed an increase in the CHOL/CER molar ratio, which was estimated to be about 1·2–1·3 : 1 in fresh scars. However, this extent of change in the CHOL/CER ratio was not so prominent when compared with the remarkably high TEWL values recorded in the early stage of fresh scars. Most importantly, we found no significant change in the CER 1 proportion in the SC lipids of fresh scars. What we found here was only a mild increase in CER 4 and CER 7 and a slight decrease in CER 3. Although we cannot speculate further about the mechanisms underlying these changes in the SC lipids of fresh scars from our present results, the unique CER profiles seem to be associated with alterations in keratinocyte differentiation due to the inflammatory changes in the upper dermis. Most of all, the results obtained strongly suggest that the barrier defect found in fresh scars is due to a mechanism other than that related to the mildly altered SC lipid profiles. We cannot exclude, however, that these changes, especially an increase in the greatly polar CER 7, may be the cause of the higher water-binding capacity of the SC in fresh scars. We cannot discuss the role of other moisturizing factors in the SC, because we did not measure their content in the present study. However, it is highly likely that the changes in amino acid and protein components of the corneocytes covering fresh scars play a role in the higher water-holding capacity of the SC of scars.

It is remarkable that TEWL recorded at the 1-month-old fresh scar still showed high levels. The normalization process of the SC barrier function at the sites of skin injury varies greatly according to the depth of initial damage; it takes only 2 weeks after removal of the SC by tape-stripping in contrast to 45 days after removal of the whole epidermis and about 1 year after dermal wounding produced by harvesting split-thickness skin grafts.[2,23] Moreover, in the case of simple removal of the SC, the initial rapid phase of recovery required for more than 70% of the basal TEWL levels takes less than 5 days.[3,23] Thus, the pattern of the recovery of the barrier function is greatly different in the case of deeper wounds which induce much more extensive and long-standing inflammation in the dermis.

Recent studies have shown the importance of CE assembly as well as lipid assembly in the SC for its competent barrier function.[24] For example, lamellar ichthyosis, which displays defective epidermal barrier function, is demonstrated to be the result of a genetic defect in the transglutaminase 1 cross-linking enzyme involved in the synthesis of CE proteins.[25–27] Retinoids are also known to suppress transglutaminase activity and to decrease CE formation.[28] It has been observed in retinoid-treated skin that abundant colloidal lanthanum tracer leaks into corneocytes, and an abnormal corneocyte fragility was speculated to account for this barrier abnormality.[29] It is also assumed that the outer lipid envelope of the CE forms a template upon which the lipid-enriched intercellular matrix is organized into lamellar structures.[30]

In normal skin, mature, polygonal rigid CEs are present only in the outer layer of the SC, whereas immature, irregularly shaped fragile CEs are usually found in the middle and deep layer of the SC.[10,31] When we studied the properties of the corneocytes in fresh scars, we found the presence of many immature and less hydrophobic CEs even in the outermost layer of the SC of fresh scars, as in irritated skin[10] and various scaly inflammatory dermatoses.[32] Scanning electron microscopic examination demonstrated that these corneocytes of the outermost layer of the SC in fresh scars looked remarkably fragile, having numerous fine wrinkles on their enlarged and flat surfaces. Most of all, in the fresh scars we found a similar, time-dependent, exponentially decreasing pattern in both the ratio of immature corneocytes with poorly hydrophobic CEs and the recorded TEWL values. Therefore, there was a highly significant positive correlation between the proportion of immature CEs in the outermost layer of the SC and the TEWL. From these observations, as also noted in retinoid-treated skin, it is much more likely that the presence of the immature

CEs is responsible for the barrier dysfunction of the SC in fresh scars than are the changes in the SC lipid profile. We can speculate from the data obtained from the SC of such fresh scars that an incomplete differentiation process in the epidermis and SC is mainly responsible for the increased appearance of immature CEs in the outermost layer of the SC. Moreover, we think that the aesthetic effects resulting from various types of chemical peeling as well as retinoids, which mildly affect the superficial portion of the dermis, also at least partially depend on similar mechanisms.

In conclusion, the permeability barrier dysfunction of fresh scars can be attributed to the formation of immature and less hydrophobic CEs rather than to the altered SC lipid composition.

Acknowledgments

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