

ORIGINAL ARTICLE

Comparison of Several Reconstructed Cultured Human Skin Models by Microscopic Observation: Their Usefulness as an Alternative Membrane for Skin in Drug Permeation Experiments

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Abstract

Several reconstructed cultured human skin models (RSMs) are already utilized as membrane alternatives to human and animal skin in skin corrosive/irritation tests. They are also utilized in skin permeation experiments from the viewpoint of animal welfare; however, different permeation profiles of chemicals were found between RSMs and excised human or animal skin. RSMs and excised human skin were morphologically evaluated by a light microscope and a transmission electron microscope. In the results, the micromorphology of all RSMs differed from that of human skin. In particular, the lamellar layer between corneocytes in RSMs was much narrower than that in the human stratum corneum. The lamella layer affects not only the diffusion and partition properties of chemical compounds in RSMs but also the concentration-distance profile of chemicals in the models. Furthermore, esterase distribution in RSMs was different to that in human skin. This difference would certainly affect the permeation of both parent ester compounds and their metabolites through RSMs. Evaluation of the morphological and enzymatic differences between RSMs and human skin would be helpful to understand the differences in the chemical permeation profiles between RSMs and human skin.

Key words: *reconstructed cultured human skin model, skin permeation, skin morphology, lamella layer*

Introduction

Reconstructed (three-dimensional) cultured human skin models (RSMs) are already used as membrane alternatives to human and animal skin in skin permeation experiments. Although the EpiskinSM (Skinethic Laboratories, St. Philippe, France) and EpiDermTM Epi606X (Kurabo Industries Ltd., Osaka, Japan) among RSMs have already been used for skin corrosive/irritation tests of chemical compounds, there are only a few reports on chemical com-

pound permeability through RSMs (Schmook *et al.*, 2001, Watanabe *et al.*, 2001, Netzlaff *et al.*, 2007).

The skin concentration of chemical compounds can be well estimated from skin permeation parameters, which are obtained from the time course of the cumulative amount of the compounds permeating skin (Sugibayashi *et al.*, 2010). Furthermore, skin irritation was found to be fairly related to the skin concentration of chemical compounds (Kano *et al.*, 2006).

These results strongly suggested that evaluation of chemical compound permeation through skin would be necessary to assess the safety of topically applied chemical compounds.

We reported that the skin permeation parameters of chemical compounds were quite different between human skin and RSMs (Kano *et al.*, 2010). Furthermore, esterase activity in skin equivalent TESTSKINTM LSE-high (Toyobo Co., Ltd., Osaka, Japan) was also different to excised rat skin (Sugibayashi *et al.*, 2004). This difference would affect not only skin permeation but also the skin concentration of ester-containing parent compounds and their hydrolyzed metabolites. In the present study, we investigated histological differences between human skin and RSMs by microscopic observation. Furthermore, carboxylesterase activities and enzyme distribution differences among RSMs were investigated to understand RSM features as an alternative animal membrane to human skin in skin permeation experiments.

Materials and Methods

1. RSMs and excised human skin

TESTSKINTM LSE-high (LSE-high), EpiDermTM Epi606X (EpiDerm), Neoderm-E, Vitrolife-skin, LabCyte EPI-model and EpiskinSM (Episkin) were purchased from Toyobo Co., Ltd., Kurabo Industries Ltd., Tego Science Inc. (Seoul, Korea), Gunze Ltd. (Kyoto, Japan), Japan Tissue Engineering Co., Ltd. (Gamagori, Aichi, Japan) and Skinethic Laboratories, respectively. All of these models were used within 3 days after receipt. All experiments were repeatedly conducted with different product lots. Excised abdominal human skin (57 years old, Caucasian female) was purchased from Biopredic International (Rennes, France). The excised human skin experiment was approved by the KAC ethics committee for human-derived products.

2. Hematoxylin and eosin staining of skin section

RSMs and excised human skin were embedded in paraffin wax and continuous sections were prepared at 3 μ m thickness using a microtome (Yamato Kohki Industrial Co., Ltd, Saitama, Japan). The specimens were sequentially im-

mersed into xylene, 100%, 90%, 80% and 70% ethanol to remove paraffin. The sections were then stained with hematoxylin and eosin (H.E.) for observation by light microscopy (IX 71; Olympus Co., Tokyo, Japan).

3. Measurement of skin thickness

The H.E.-stained skin sections were observed using a light microscope equipped with a digital camera (DP 72; Olympus Co.), and the thickness of skin sections was measured using DP2-BSW software (Olympus Co.).

4. Observation of lamella structure with a transmission electron microscope

Lamella structures in RSMs were observed by a transmitted electron microscope (TEM) (JEM1200-Ex; Jeol Ltd., Tokyo, Japan). Excised human skin and RSMs were fixed with 0.1% phosphate buffer containing 2% paraformaldehyde and 2% glutaraldehyde, followed by 2 % osmium tetroxide, then with 0.2% ruthenium tetroxide/0.25% potassium hexacyanoferrate. The fixed specimens were embedded in epoxy resin. The skin samples were sectioned at 80–90 nm thickness using an ultramicrotome. The obtained sections were observed by a TEM after double staining with uranyl acetate and lead citrate.

5. Observation of skin structure with an *in vivo* confocal laser scanning microscope.

Noninvasive observation of the internal human skin structure was performed *in vivo* by a confocal laser scanning microscope (CLSM) (Vivascope 1500; Lucid Inc., Rochester, NY, U.S.A.) (Sauermann *et al.*, 2002, Hegyi *et al.*, 2009). In the present experiment, RSMs and human skin were observed every 1 μ m until 50 μ m depth from the membrane surface. The Vivascope 1500 was equipped with an 830 nm wavelength diode laser and water-immersion objective lens ($\times 30$). Skin images of 500 $\times$ 500 μ m were obtained at each observation.

6. Evaluation of skin distribution of esterase enzymes

RSMs and excised human skin were rinsed with pH 7.4 phosphate-buffered saline (PBS) and embedded into Tissue-Tek[®] OTC compound

(Sakura Finetek Japan Co. Ltd., Tokyo, Japan). These samples were frozen in cold isopentane (2-methyl-butan) at -20 °C. Frozen sections, 20 µm thick, were prepared by a cryostat (CM3050S; Leica Microsystems Ltd., Heerbrugg, Switzerland) and the specimens were observed using a fluorescence microscope (CK40; Olympus Co.) 15 min after application of 1 mL of 0.02 mg/mL fluorescein-5-isothiocyanate diacetate (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The excess solution was washed off with pH 7.4 PBS before observation.

Results

1. Comparison of H.E.-stained skin images

Table 1 shows the thicknesses of RSMs and excised human skin. Each RSM ($13.2 \pm 4.6 \sim 89.0 \pm 1.0$ µm) had a thicker stratum corneum than human skin (11.5 ± 6 µm). On the other hand, a thinner viable epidermis and dermis in RSMs ($23.3 \pm 4.6 \sim 110.1 \pm 4.1$ µm) was observed than in human skin (474 ± 7.5 µm). Figure 1 compares H.E.-stained images of excised human abdominal skin (Fig. 1a) and RSMs (Fig. 1b-g). Widely separated stratum corneum layers were observed in several H.E.-stained RSMs compared to human skin. The number of

cell nuclei stained by hematoxylin in each RSM was lower than in human skin, especially in LSE-high (Fig. 1b) and Neoderm-E (Fig. 1f). Interestingly, many hematoxylin-stained cell nuclei were observed in the stratum corneum in Vitrolife skin (Fig. 1e). This may have been due to inadequate keratinization of keratinocytes.

2. Comparison of lamella structure in stratum corneum observed by a transmission electron microscope

Figure 2 shows TEM images of excised human skin (Fig. 2a) and RSMs (Fig. 2b-g). Lamella layers were frequently observed in human skin (Fig. 2a). Although lamella layers were also found in RSMs (Fig. 2b-g), the frequency was relatively lower than in human skin. The lamella layers in LSE-high (Fig. 2b) were narrow and tortuous compared to those in human skin. EpiDerm showed a fairly similar structure (Fig. 2c) to human skin. Neoderm-E (Fig. 2f), Lab-Cyte EPI-model (Fig. 2d) and Episkin (Fig. 2g) have aggregated lipid structures and localized lamella analog layers in their intercellular spaces, whereas no lamella layers were observed in Vitrolife skin (Fig. 2e).

Table 1 Skin thickness of excised human skin and reconstituted cultured human skin models.

	Stratum corneum	Viable epidermis (µm)	Dermis (µm)	Whole skin (µm)
Human	11.5 ± 0.57 (11.8 ± 3.3) ^{#)}	62.4 ± 3.45 (51.2 ± 12.2) ^{#)}	412 ± 24.0	493 ± 24.6
LSE-high	29.9 ± 0.93	29.2 ± 2.08	80.8 ± 2.05	136 ± 136
Epiderm	27.0 ± 0.71 12-28 ^{*)}	53.0 ± 1.15 28-43 ^{*)}	---	78.9 ± 78.9
Labcyte EPI-model	89.0 ± 1.00	59.1 ± 0.60	---	149 ± 1.07
Vitrolife skin	13.2 ± 4.61	23.25 ± 4.61	---	38.6 ± 5.23
Neoderm-E	27.8 ± 0.93	23.8 ± 0.87	---	52.2 ± 0.720
Episkin	69.3 ± 1.04 79-102 ^{*)}	28.7 ± 0.92 38-48 ^{*)}	---	99.2 ± 2.58

* Ponc et al., 2000.

Sato et al., 1991.

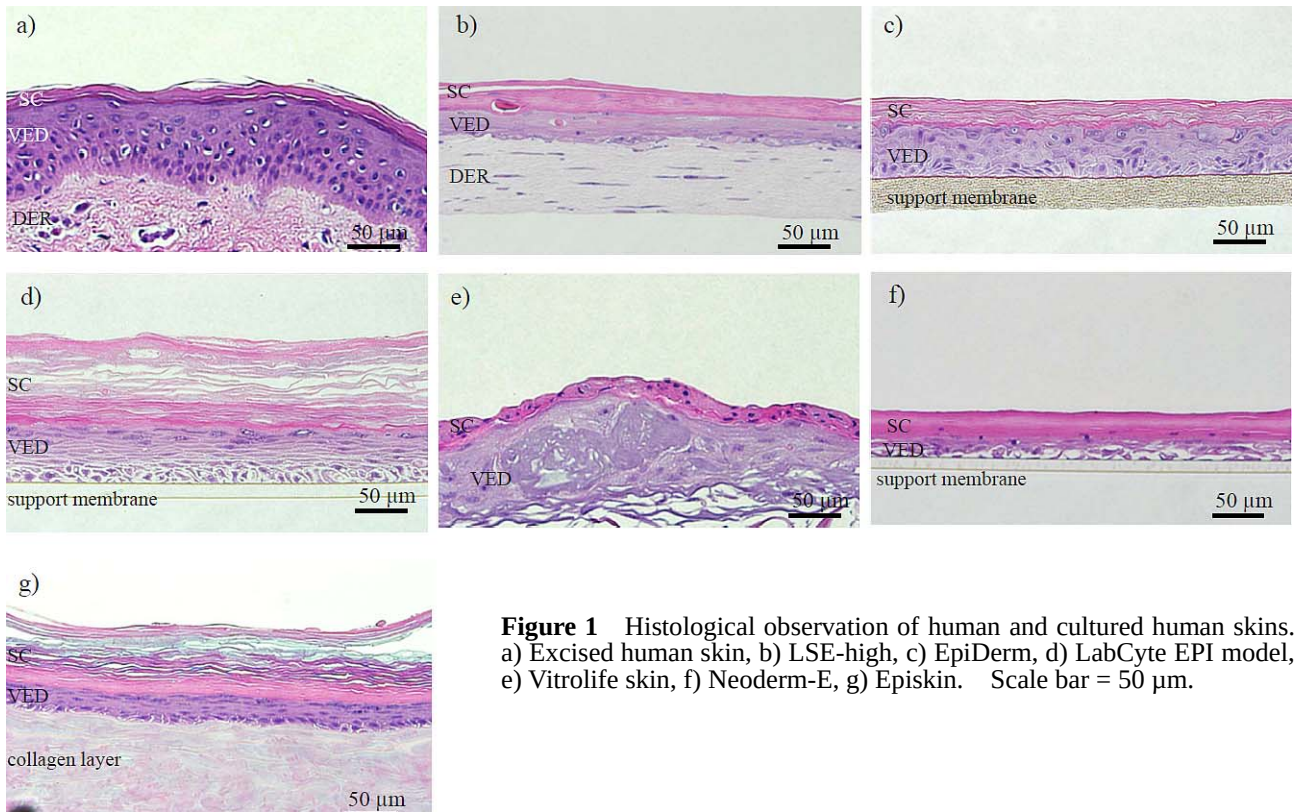


Figure 1 Histological observation of human and cultured human skins. a) Excised human skin, b) LSE-high, c) EpiDerm, d) LabCyte EPI model, e) Vitrolife skin, f) Neoderm-E, g) Episkin. Scale bar = 50 μm.

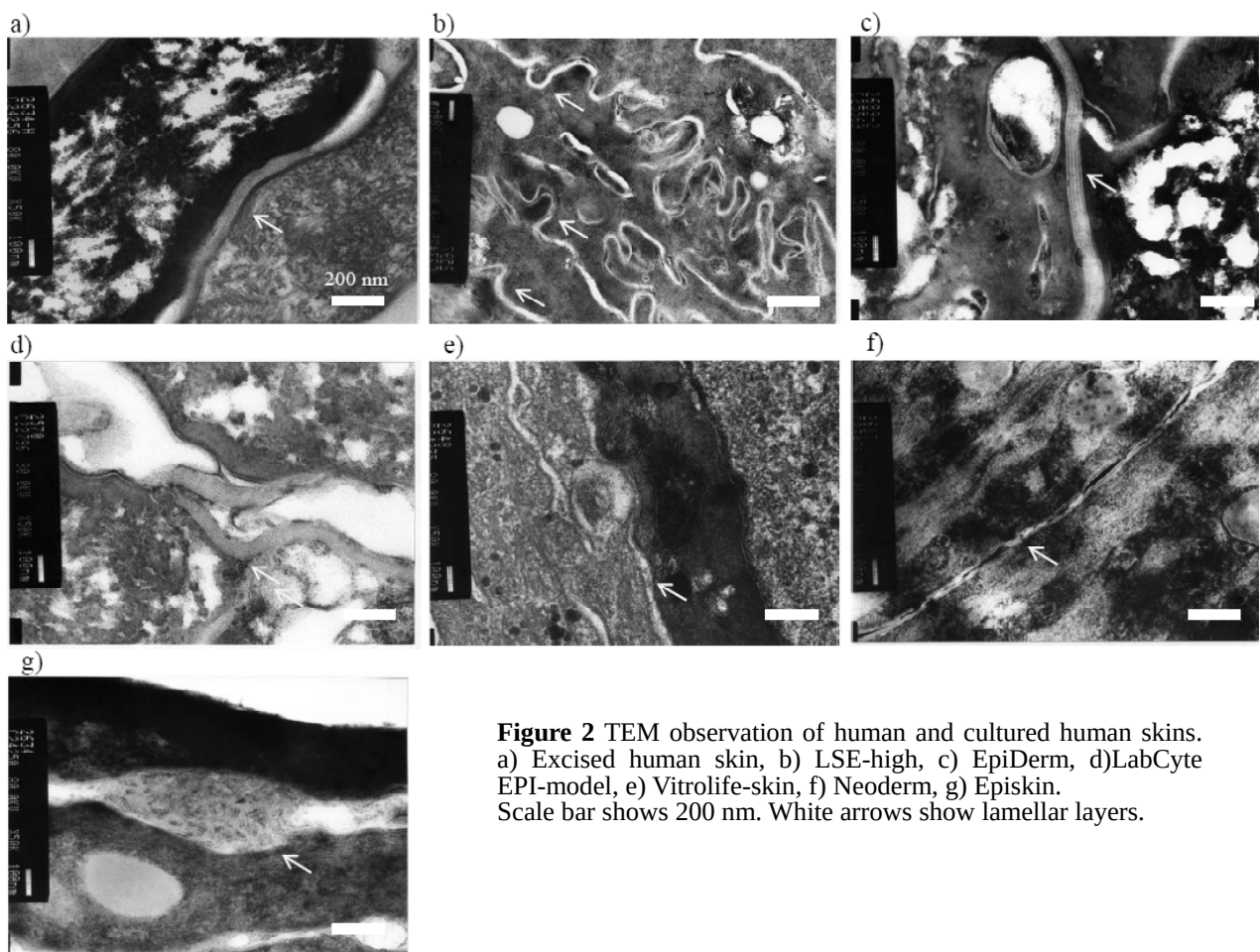


Figure 2 TEM observation of human and cultured human skins. a) Excised human skin, b) LSE-high, c) EpiDerm, d) LabCyte EPI-model, e) Vitrolife-skin, f) Neoderm, g) Episkin. Scale bar shows 200 nm. White arrows show lamellar layers.

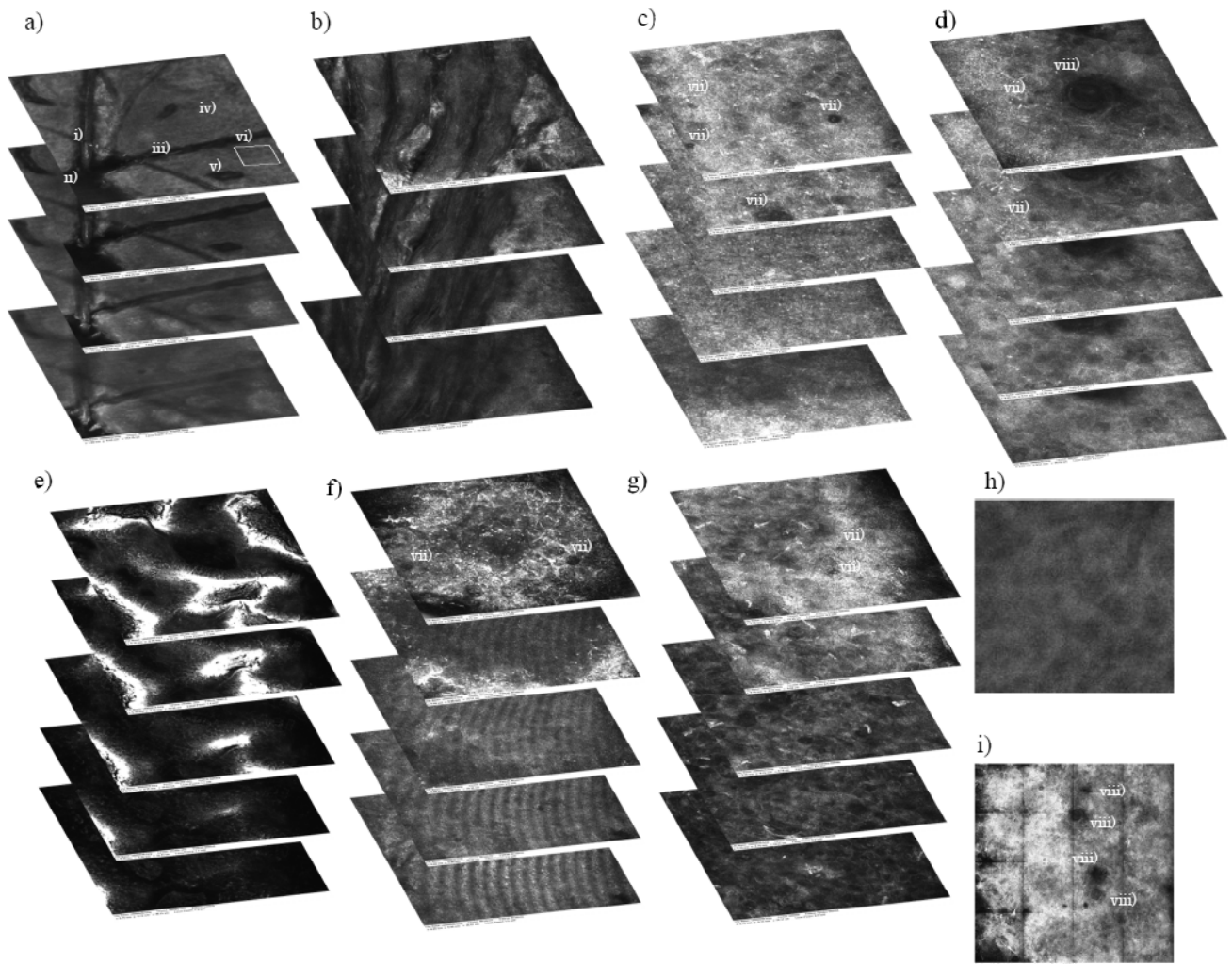


Figure 3 TEM observation of human and cultured human skins. a) Excised human skin, b) LSE-high, c) EpiDerm, d) LabCyte EPI model, e) Vitrolife skin, f) Neoderm-E, g) Episkin, h) Enlargement of vi) area, i) 500 × 500 μm skin images of LabCyte EPI model. Scale bar = 200 nm. i) hair, ii) hair follicle, iii) sulcus cutis, iv) crista cutis, v) sweat gland, vi) corneocyte, vii) corneocyte with nucleus, and viii) pore.

3. Observation of skin structure by an *in vitro* confocal laser scanning microscope

Figure 3 shows the *in vitro* confocal laser scanning microscope (CLMS) images of human skin (Fig. 3a) and RSMs (Fig. 3b-g). A hair (i), hair follicle (ii), sulcus cutis (iii), crista cutis (iv) and pore (v) were observed in human skin (Fig. 3a) and their sizes gradually diminished by increasing the skin depth. Furthermore, corneocytes ((vi) in Fig. 3a and an enlargement, Fig. 3h) were clearly observed in the shallow part of the human skin (Fig. 3a, h). On the other hand, the sulcus cutis and crista cutis were not confirmed in RSMs (Fig. 3b-g), but the obtained images were different from each other. Corneocytes

with a nucleus (vii) were observed in the shallow part of EpiDerm (Fig. 3c), LabCyte EPI model (Fig. 3d), Neoderm-E (Fig. 3f) and Episkin (Fig. 3g), whereas corneocytes were not clearly observed in LSE-high (Fig. 3b) and Vitrolife skin (Fig. 3e). Interestingly, pores (viii) in the skin surface were observed in the LabCyte EPI model (Fig. 3d). The pores were observed until 50 μm from the skin surface in the LabCyte EPI model (Fig. 3d). The surface image (2 mm×2 mm) of the LabCyte EPI model revealed several pores (Fig. 3i). A similar tendency was observed in different lots of the LabCyte EPI model (data not shown).

4. Comparison of esterase distribution between human skin and RSMs.

Figure 4 shows the skin distribution of fluorescein-5-isothiocyanate, a metabolite of fluorescein-5-isothiocyanate diacetate by skin esterase after topical application of fluorescein-5-isothiocyanate. A high intensity of green fluorescence was locally observed in the viable epidermis of excised human skin (Fig. 4a), suggesting that skin esterase scarcely existed in the stratum corneum, but extensively in the viable epidermis. LSE-high, EpiDerm and Episkin showed similar green fluorescence distributions to human skin (Fig. 4b, c and f). On the other hand, the LabCyte EPI model and Vitrolife skin showed green fluorescence not only in the viable epidermis but also in the stratum corneum (Fig. 4d and e).

Discussion

H.E.-stained skin sections revealed that the skin thicknesses of the stratum corneum, viable epidermis and dermis in the RSMs were different to those in the human skin. The outmost layer of skin, the stratum corneum, is the biggest barrier against the entry of exogenous substances, so its thickness might affect the skin permeation rate of chemical compounds (Barry *et al.*, 1983). Although RSMs have a thicker stratum corneum than excised human skin, RSMs generally show faster skin permeation of chemical compounds (Kano *et al.*, 2010). The shallow part of the stratum corneum in RSMs might not have an effective barrier function as in the human stratum corneum.

TEM observation and CLSM results show that RSMs have a unique structure in the inter-corneocytes. Partition and diffusion param-

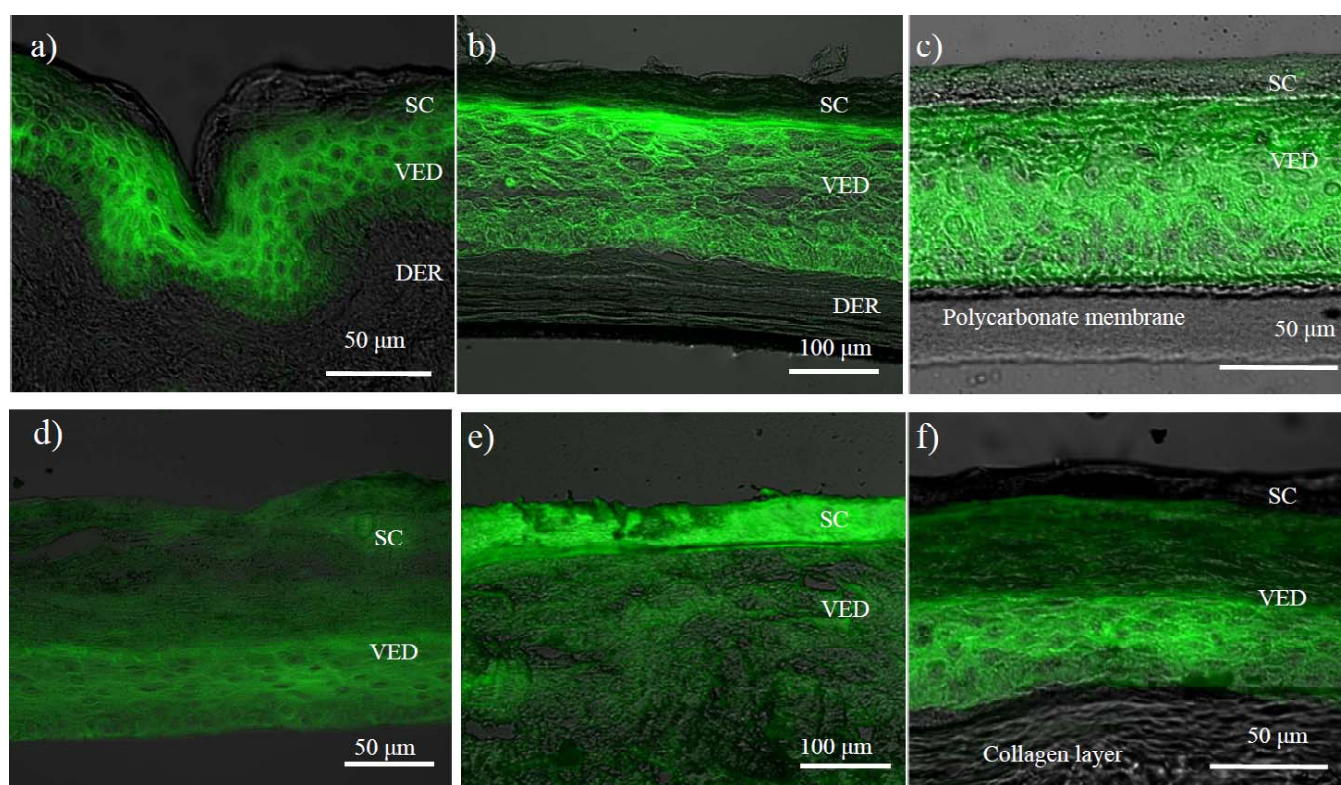


Figure 4 Fluorescein observation of human and cultured human skins 15 min after application of fluorescein-5-isothiocyanate diacetate. a) Excised human skin, b) LSE-high, c) EpiDerm, d) LabCyte EPI model, e) Vitrolife skin, f) Episkin. Scale bar = 200 nm. Abbreviations: SC: stratum corneum, VED: viable epidermis, DER: dermis.

ters of chemical compounds topically applied to RSMs were much different from human skin (Kano et al., 2010). The diffusion and partition parameters depend on the diffusivity of the chemical compounds in the stratum corneum and lipophilicity of the stratum corneum, respectively. Inter-corneocyte space is filled with ceramides and other lipids to compose the lamella structure (Elias et al., 1975, Lampe et al., 1983). Therefore, the maturity of the lamellar structure and content of lipid or constitution of intercellular lipids in the stratum corneum would affect the diffusion and partition parameters of topically applied compounds. The localized distribution and frequency of lamella layers were observed in several RSMs. These results might also relate to the different partition parameters in RSMs. Furthermore, observation using the Vivascope 1500 revealed pores in several RSMs. These RSMs might be difficult to use in skin permeation experiments in the present state because these models have different lipophilicity to human skin. The results were not fully analyzed with a small-angle X-ray diffraction analyzer in the present study. Further experiments will be needed to elucidate the lamella structure differences between excised human skin and RSMs.

Investigation of the total amount of metabolic enzymes (Luu-The et al., 2009, Hu et al., 2010) and their distribution in skin is very important because the difference in the metabolic enzyme activity and distribution between human skin and RSMs would affect not only the skin distribution of metabolites but also to their efficacy or toxicity; however, only a few studies have been carried out on the enzyme distribution in RSMs. Thus, evaluation of enzyme distribution is necessary in RSMs. The skin distribution of fluorescein-5-isothiocyanate revealed that each RSM had a different enzyme distribution, suggested that the parent and its metabolite concentration-distance profiles in each RSM would be different. Esterase is expressed in the granular layer of the epidermis (Clark et al., 1993, Jewell et al., 2007, Zhu et al., 2007); however, the Vivascope 1500 revealed denuded corneocytes in the shallow part of EpiDerm, the Lab-Cyte EPI model Neoderm-E and Episkin. These undifferentiated cells would affect esterase activity. Evaluation of the amount of esterase ac-

tivity in RSMs will be needed to use them as a membrane alternative to skin in skin permeation experiments.

Conclusion

The present histological observation suggested that structural differences in RSMs could be the reason for skin permeation differences among RSMs; however, chemical compound permeation through human skin could be predicted from rat or porcine skin despite their different structures. Therefore, combination analysis of skin permeation and histological results would be extremely effective to clarify the characteristics of each RSM. We believe that understanding the characteristics of RSMs is very important for more widespread usage of RSMs.

Acknowledgements

Preparation of skin specimens and TEM observations were conducted by Hanaichi Ultrastructure Research Institute (Okazaki, Aichi, Japan). H.E.-stained samples were prepared by Genostaff Co., Ltd. (Tokyo, Japan). Part of the present study was supported by a Grant-in-Aid for Scientific Research (H22-iyaku-ippan-002) from the Ministry of Health, Labor, and Welfare, Japan.

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(Received: May 18/
Accepted: October 25)

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