Stratum Corneum Lipids of Human Epidermal Keratinocyte Air-Liquid Cultures: Implications for Barrier Function

Alane H. Kennedy,[1] Guia M. Golden,[1] Carol L. Gay,[2] Richard H. Guy,[2] Michael L. Francoeur,[1] and Vivien H. W. Mak[3]

Received February 16, 1996; accepted April 26, 1996

Purpose. The purpose of this study is to investigate the permeability barrier, i.e., the stratum corneum (SC) lipids, of human epidermal keratinocyte air-liquid cultures and compare them with those of human SC.

Method. The SC lipids composition was analyzed by TLC technique, the organization by electron microscopic procedure, and the phase transition temperature by Infrared spectroscopic method.

Results. Electron microscopy demonstrated that The SC lipids of cultures were largely retained inside the corneocytes, and that the intercellular lipids lack both the basic unit repetition (*i.e.*, broad : narrow : broad : broad : narrow : broad of electron lucent bands) and the covalently-bound lipid envelope normally found in human SC. These characteristics are similar to those found in SC from patients with atopic dermatitis or psoriasis, or from animals with essential fatty acid deficiency, suggesting that the cultures may be hyperproliferative. In addition, the high free sterol content and the altered fatty acid/ceramide composition of these cultures argue that the compromised barrier function is linked to hyperproliferation and lipid synthesis, or vice versa. Infrared spectroscopic analyses confirm that there are major conformational differences between the lipids of human and cultured SC.

Conclusions. The profound differences in SC lipid composition, organization and conformational properties attest that permeability alone is not a sufficiently sensitive marker to define barrier equivalence between cultures and human skin.

KEY WORDS: keratinocyte cultures; skin barrier function; stratum corneum lipids.

INTRODUCTION

To date, several attempts have been made to validate skin cultures for permeability, pharmacology, toxicity and metabolism studies, and to generally gauge the relative extent to which these systems are equivalent to *in vivo* human skin. These includes the overall morphology, the pattern of keratin expression, and measurements of water permeability. In this regard, earlier results from our laboratory indicated that the particular culture system described in this paper yielded epidermal samples which were histologically (ascertained by conventional hematoxylin and eosin staining) very similar to human epidermis, and that water flux was only 2–3 fold greater than that found with intact skin (1). Intuitively, good morphology and small differences in water permeability would suggest that these human A/L cultures are fairly representative of *in vivo* human epidermis; especially since the final product of terminal keratinocyte differentiation is the stratum corneum (SC), which dictates the permeability properties of skin (2,3).

The purpose of this study is to establish whether keratinocyte cultures truly develop a permeability barrier equivalent to *in vivo* human epidermis. We have undertaken extensive structural and compositional analyses of SC samples obtained from our culture system. By using various analytical and biophysical techniques, we have determined the extent to which the lipid structural, conformational and compositional properties of cultured SC resemble those of human SC.

MATERIALS AND METHODS

Culture

The culture technique has been described in detail elsewhere (1). In brief, first-passaged neonatal human keratinocytes were plated on de-epidermized human dermis at 0.5×10^6 cells/cm^2. The cultures were grown submerged at 37°C and 95% air/ 5% CO_2 for three days before lifting to the A/L interface. These keratinocyte cultures were nourished with Dulbeccos' Modified Eagle Medium (high glucose, Gibco Lab., Life Technologies, Inc., Grand Island, NY) supplemented by human serum (10%, Gibco), insulin (5μg/ml; Gibco), hydrocortisone (0.4 μ/ml; Aldrich Chemicals) and cholera toxin (0.5 μg/ml; Sigma, St. Louis, MO). Cultures were exposed to 75% r.h. at the A/L interface for three weeks before further examination by the techniques described below. Human skin was obtained from human cadavers (upper thigh) or following surgery (breast). SC from human skin (referred to as human SC) or from cultured epidermis (referred to as cultured SC) was prepared following trypsinization at room temperature for one hour as described previously (4).

Electron Microscopy

Human skin or keratinocyte culture samples were minced to approximately 1 mm^3 pieces, placed in 2% glutaraldehyde, and stored at 4°C overnight. Samples were then rinsed in 0.1 M cacodylate buffer, and post-fixed in 0.2% ruthenium tetroxide for two hours at 4°C. After further rinsing in 0.1 M cacodylate buffer, samples were dehydrated using a graded series of acetone solutions and embedded in Spurr's resin. Thin sections of the samples were stained with uranyl acetate and lead citrate before examination on a Zeiss 109 electron microscope.

Lipid Analyses

Precoated silica gel high-performance thin-layer chromatographic plates, 10 × 20 cm, (HP-TLC, E. Merck, Darms-

[1] Dermal Therapeutics Group, Central Research Division, Pfizer Inc, Groton, Connecticut 06340.

[2] Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143-0446.

[3] To whom correspondence should be addressed at: Cellegy Pharmaceuticals, Inc., 871 Industrial Road, J & K, San Carlos, California 94070.

ABBREVIATIONS: SC, stratum corneum; MCG, membrane coating granules; r.h., relative humidity; EM, electron microscopy; HP-TLC, high-performance thin layer chromatography; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared spectroscopy; A/L, air/liquid; EFAD, essential fatty acid deficiency; TEWL, transepidermal water loss; ^3H_2O, tritiated water; DPPC, dipalmitoylphosphotidylcholine; AD, atopic dermatitis.

0724-8741/96/0800-1162$09.50/0

tadt, Germany) were prewashed by developing overnight in chloroform:methanol:water (95:20:1) and dried before use. SC lipids (150 μg) or standards (2.5 μg) were applied to the plates in 5 μl of chloroform:methanol (2:1). Sequential development of the plates was used to achieve separation of all lipid classes. The developing procedure was: a) chloroform:methanol:water (40:10:1) to 6 cm from the origin, b) chloroform:methanol:glacial acetic acid (190:9:1) to 10 cm from the origin, c) hexane:diethyl ether:glacial acetic acid (8:2:1) to 13 cm from the origin, and d) petroleum ether to 17 cm from origin. Finally, visualization was accomplished by spraying with 10% (w/v) cupric sulfate in 8% phosphoric acid, and heating to 180°C for 30 minutes.

FTIR Spectroscopy

Infrared spectra were obtained using a Nicolet (Madison, WI) Fourier transform instrument equipped with a liquid N_2-cooled Mercury-Cadmium-Telluride-B detector. For each spectrum, 200 scans were co-added and transformed with a Happ-Genzel apodization function to yield a final resolution of 2 cm^{-1}. The stratum corneum samples were sandwiched between ZnS windows mounted in a specially designed heating/cooling cell. This cell was connected to a low-temperature water circulator containing a mixture of 50% ethylene glycol-water. The water bath and, ultimately, the sample temperature were controlled by a separate computer interfaced to the Nicolet workstation. Using a thermocouple, which was in direct contact with the sample to monitor temperature, the spectra were collected at 2 (± 0.1) °C increments at a rate of 20°C/hr. Peak positions were measured with a center of gravity algorithm which allowed frequencies to be determined with an uncertainty of less than 0.05 cm^{-1}.

RESULTS AND DISCUSSION

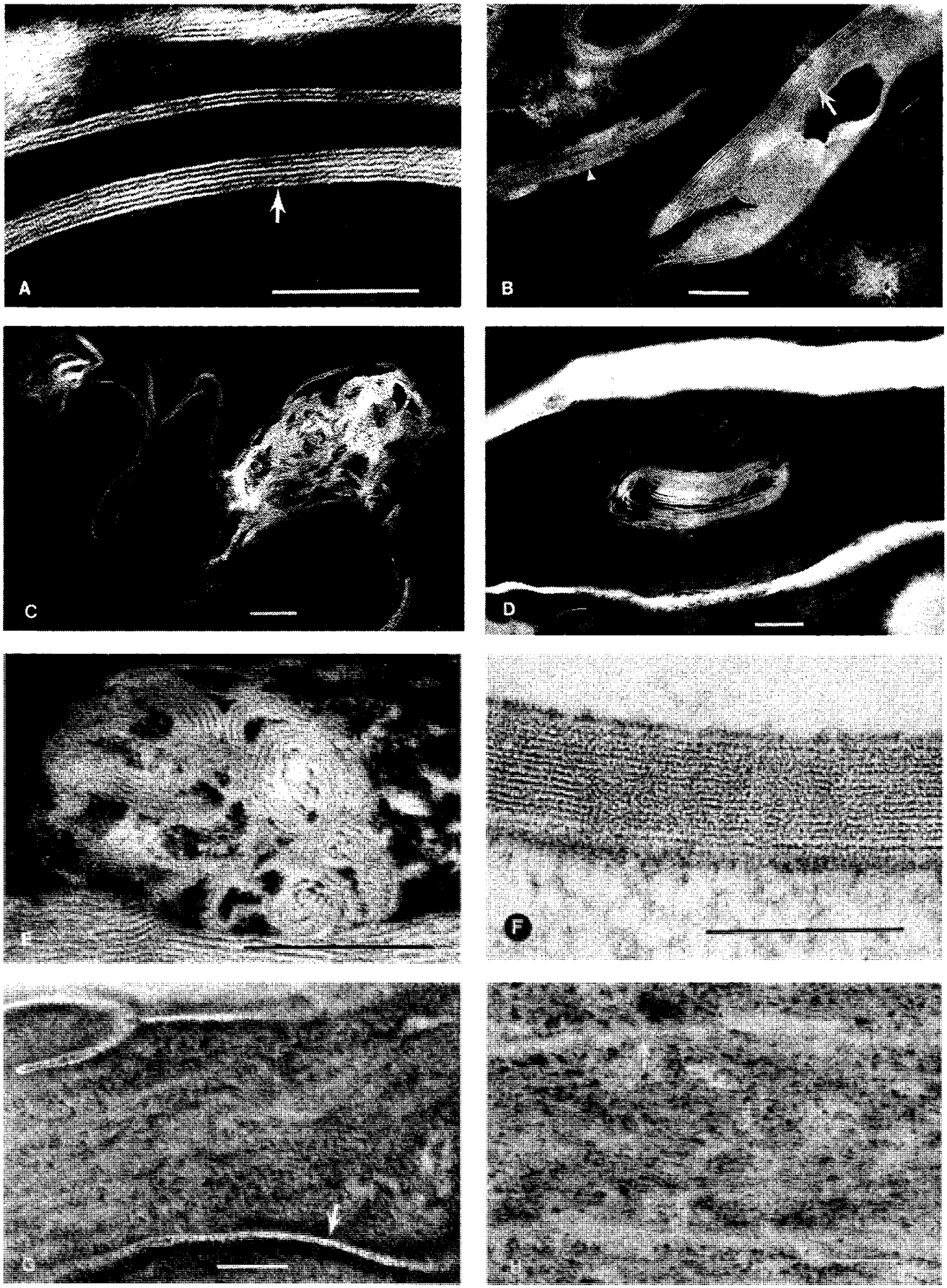
The overall patterns of lipid organization for various cultured and human SC samples are presented in the electron micrographs shown in Figures 1A to 1G. In human SC, a specific intercellular lamellar lipid pattern was observed (Figure 1A) in each of the samples obtained from four individual donors. The basic unit comprises bands which are, sequentially broad : narrow : broad : broad : narrow : board in width (*i.e.* BNBBNB) (5). Similar intercellular lipid patterns have also been found in pig and hairless mouse (5,6). It is important to realize that this pattern does not exist throughout the intercellular spaces (Figure 1B). Further, lamellar lipids also exist in the cytosol of human SC corneocytes (Figure 1B).

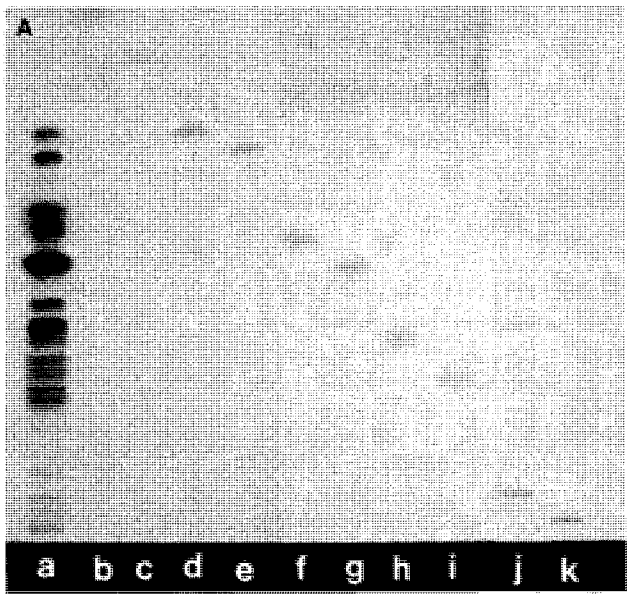
Cultured SC lipids, on the other hand, are much less uniformly distributed than those in human SC (Figure 1C) containing only 6% extractable lipids comparing with 10–15% for human SC. The poorly organized lipids of cultured SC are characterized by a significant amount of *intracellular* lamellar structures (Figure 1C) appearing as either onion-like bilayer structures (Figure 1D) (7), or partially extruded lipids (Figure 1E) (8–10). Nowhere in the cultured SC are there bilayer structures in the BNBBNB pattern seen in human SC, instead, the *intercellular* lipids present lucent bands of approximately equal width. Analogous bands of uniform width (Figure 1F) have also been observed in the SC of essential fatty acid deficient (EFAD) hairless mice (6), in porcine SC which has been heated to 80°C, and in human cysts (5). There were also numerous areas of the cultured SC in which only a single lucent band adjacent to the corneocyte was observed. In human or porcine SC, this lucent band is a covalently-bound monolayer of N-(ω-hydroxyacyl) sphingosines (11) which cannot be removed by the present delipidization procedure (Figure 1G). In contrast, this band is completely removed from the cultured SC (Figure 1H) implying that the covalent lipid envelope is not formed in these cultures. The organization of cultured SC lipids is also reminiscent of the barrier layer in psoriatic epidermis (12), or in skin obtained from patients with atopic dermatitis (AD) (13), which are hyperproliferative skin conditions characterized by an atypical SC and a compromised water barrier.

The HP-TLC results for the cultured and human SC are shown in Figures 2A and 2B. Since equivalent amounts of lipid from cultured and human SC were applied onto the silica plates, the distribution of the individual lipids can be qualitatively compared by the relative intensities (Figure 2A). All the major classes of SC lipids, including both polar (ceramides and cholesterol sulfate) and non-polar subsets (free sterol, free fatty acids, triglycerides and cholesterol esters) (Figures 2A and 2B) were produced by the human keratinocyte A/L cultures. However, a higher free sterol content (both cholesterol and lanosterol which migrates above cholesterol) was found in the cultured SC (Figure 2B). Interestingly, enhanced epidermal sterol biosynthesis has also been demonstrated in EFAD mice (14), and in psoriatic (15) and AD lesions (16). Given that psoriatic (17), EFAD (14) and AD skin (18) also present a compromised barrier to water, it follows that increased free sterol content in culture could contribute to the incomplete barrier function. In addition to enhanced sterol levels, a *reduced* free fatty acid content (especially those with C_{22}–C_{28} alkyl chains) was found in the cultured SC, similar to that found in human SC treated with sodium dodecyl sulfate (19). Other parallels between the cultured and surfactant-treated skin are hyperproliferation, an altered production of ceramides, and a higher TEWL. Since both psoriatic and EFAD epidermis also have an *increased* fatty acid content, the common denominator of these pathological skin conditions, i.e. a hyperproliferative or compromised barrier alone, does not appear to be the sole contributing factor to the reduced longer-chain fatty acid quantities in cultured SC.

Another critical question with regard to lipid composition pertains to the lowered level and abnormal distribution of ceramides in the cultured SC, relative to human SC. These differences may be confounded by the fact that cultured SC extracts probably include ceramides which normally constitute the corneocyte lipid envelope. Although ceramides are unequivocally crucial to the SC barrier function (20), the profound differences between the cultured SC and human SC (over and above the ceramides alone) could collectively contribute to the previously observed higher value in water permeation (1).

Figures 3A and 3B show the temperature-dependent changes in the CH_2 symmetric stretching frequency (υ_{CH_2}), measured by FTIR, for human SC and cultured SC, respectively. Lipid phase transition temperatures can be deduced from the inflection points of these curves, or from the maxima in the standard deviations of the mean frequency values (Figures 4A & 4B). Human SC exhibits four thermal transitions, three lipid-associated (*i.e.*, @~40, 70 & 80°C) and one protein-related (*i.e.*, @~95°C) (21,22). FTIR spectroscopy, like DSC, can also be used to monitor phase transitions providing additional infor-


A
B
C
D
E
F
G
H



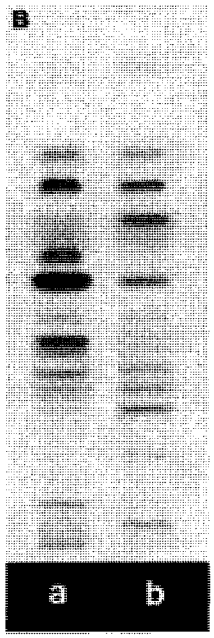
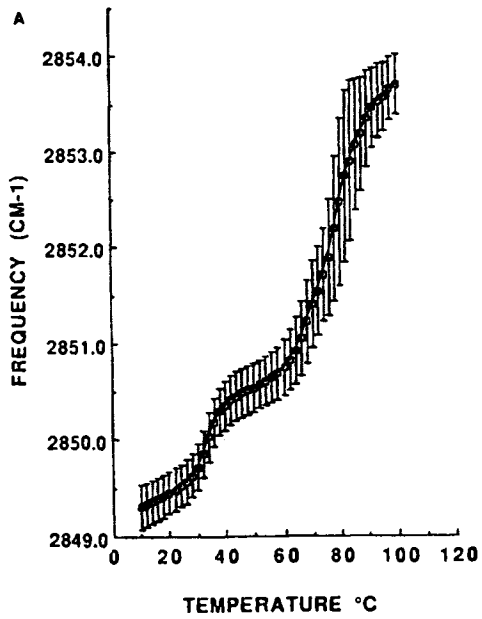


Fig. 2. Stratum corneum lipid composition separated by HP-TLC plates with the solvent systems described in the Method section; (A) with standards, a: SC lipids, b: hydrocarbon (eicosane), c:squalene, d:cholesterol oleate, e:triglyceride, f:fatty acids (palmitic acid), g:cholesterol, h:ceramides III(bovine brain), i:ceramides IV(bovine brain), j:cholesterol sulfate, and k:phospholipids, and (B) a: **cultured,** and b: **human** SC lipids.



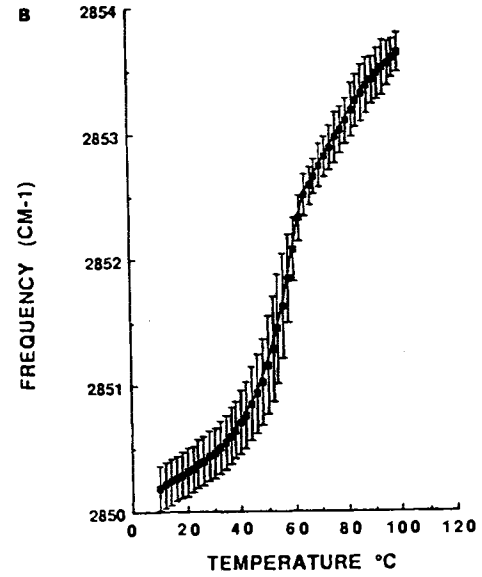


Fig. 3. (A) **Human SC** lipid transitions indicated by CH_2 stretching frequency changes as a function of temperature (n = 5, ave ± sd). (B) **Cultured SC** lipid transitions indicated by CH_2 stretching frequency changes as a function of temperature (n = 5, ave ± sd).

mation at the molecular level. FTIR is particularly useful for SC structural analysis since the CH_2 vibrational stretching frequency (υ_{CH_2}) is sensitive to the average conformational order of the SC intercellular lipids; υ_{CH_2} increases as disorder

Fig. 1. Stratum corneum lipid structure revealed by EM following ruthenium tetraxide post-fixation; (A) **human** SC intercellular lipids with the basic unit (-BNBBNB-) pattern (arrow), (B) **human** SC with non-lamellar intercellular lipids (arrow) and intracellular lipids (arrowhead), (C) **cultured** SC, note the poorly organized lipid structures, (D) **cultured** SC intracellular lipids showing an onion-like structure, (E) **cultured** SC lipid structure resembling that seen during the membrane coating granule extrusion process, (F) **cultured** SC intercellular lipids with electron lucent bands of approximately equal width, (G) **delipidized human** SC showing the retention of the covalently bound lipid envelope (arrow), and (H) **delipidized cultured** SC, note the absence of a bound lipid envelope (arrow). Bars, 0.1 μM from IA–1H.

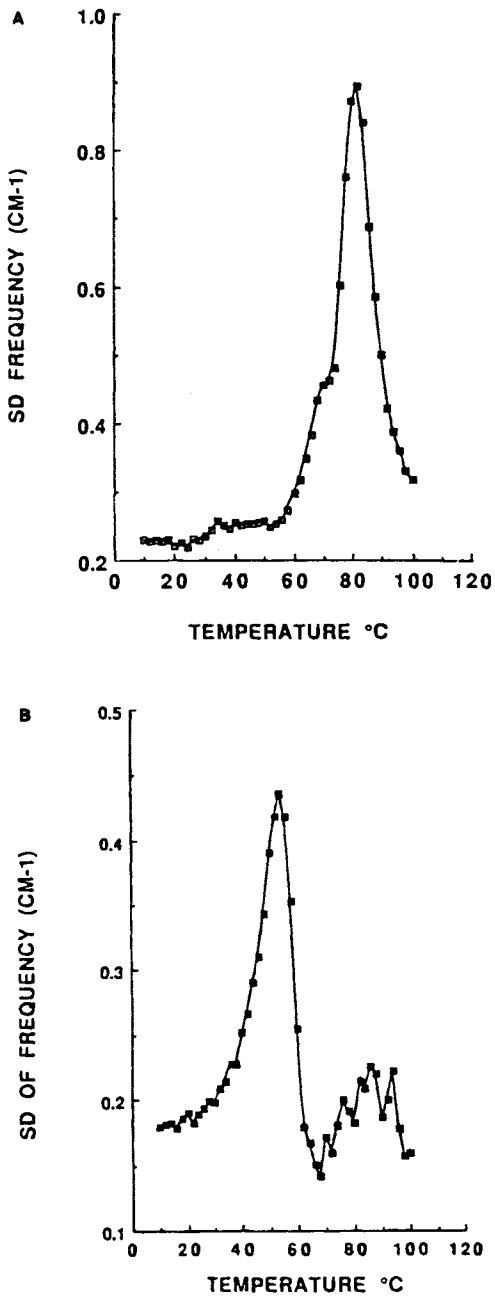


Fig. 4. (A) The standard deviations of **Human SC** CH_2 stretching frequency as a function of temperature. (B) The standard deviations of **Cultured SC** CH_2 stretching frequency as a function of temperature.

increases. Inspection of the temperature-induced changes in the CH_2 stretching frequency from human SC (Figures 3A and 3B) clearly revealed three transitions at approximately 35,70 and 80°C. For human SC, the relatively large increase in υ_{CH_2} between 50 and 100°C is indicative of a gel-to-liquid crystalline phase transition involving lipid alkyl chain melting (21). The existence of two lipid phase transitions in the 70–80°C range implies the melting of two distinct populations of lipid alkyl chains. It has been demonstrated with dipalmitoyl phosphotidylcholine (DPPC) in that the introduction of cholesterol results in a separation of cholesterol-rich and cholesterol-free lipid populations (23). Consequently, a reasonable explanation for the high-temperature transitions is that two distinct lipid subsets are partially derived from the polar SC lipids (mainly ceramides), one with cholesterol and one without.

The temperature dependence of υ_{CH_2} of the cultured SC was quite different, indicating a major phase change at 50°C, and the suggestion of a small transition near 80°C (Figures 3B and 4B). The occurrence of a main phase change at 50°C for the cultured SC, may reflect a depression of the 70°C transition observed for human SC. Given the lipid compositional differences observed by TLC, we suggest that this suppression may be partly due to the elevated presence of cholesterol (and/or lanosterol) which would tend to reduce the transition temperature (24,25), whereas the suggestion of a small 80°C transition is consistent with the absence of bound lipid envelope seen in the electron-micrograph of the cultured SC.

While the low temperature transition (~40°C) has been tentatively ascribed to sebaceous lipids (21), our TLC data (Figure 2B) do not support this assignment. We suggest that this is likely a solid-to-fluid phase change involving a small subset of the endogenous SC lipids. Corroborative evidence for the existence of this fluid lipid phase in hairless mouse SC and porcine lipids at physiological temperature has been derived from x-ray diffraction and NMR spectroscopy, respectively (26,27). The cultured SC, unlike its human counterpart, did not exhibit a low temperature lipid transition. While the lipids responsible for this low temperature transition can not be specifically identified, those which are usually regarded as nonpolar (*e.g.*, triglycerides, cholesterol esters, *etc.*) would appear to be the likely candidates. A second, and perhaps more plausible explanation, is that the cultured SC lipids may be physically organized and segregated differently to those in normally-differentiating human SC. This idea is consistent with the EM results that cultured and human SC differ significantly in the appearance of lipid organization.

The consistent message from the EM, TLC and FTIR results presented here is that, under the present conditions, our *in vitro* skin culture does not generate a SC equivalent to human SC and could, in fact, be hyperproliferative. What is unclear at this point is whether it is the hyperproliferation which leads to a compromised barrier or vice-versa, since compromised barrier function is known to enhance lipid biosynthesis (28) and to upregulate DNA synthesis (29,30). Since permeability is controlled by physical-chemical (partition and penetration) and biochemical (proliferation, differentiation, metabolism, *etc.*) factors, it is clear that one should not use the permeability data alone, or regular histological examination (hematoxylin & eosin), to gauge the performance of any given *in vitro* culture system. It is thus essential that any system prepared be fully characterized by multiple criteria before it is used to replace human epidermal tissue.

REFERENCES

1. V. H. W. Mak, M. B. Cumpstone, A. H. Kennedy, C. S. Harmon, R. H. Guy, R. O. Potts. Barrier function of human keratinocyte cultures grown at the air-liquid interface. *J Invest Dermatol.* **96**:323–327 (1991).
2. R. J. Scheuplein and I. H. Blank. Permeability of the skin. *Physiol Rev* **51**:701–747 (1971).
3. W. J. Albery and J. Hadgraft. Percutaneous absorption: in vivo experiments. *J Pharm Pharmacol* **31**:140–147 (1979).

4. G. M. Golden, D. B. Guzek, A. H. Kennedy, J. E. McKie, R. O. Potts. Stratum corneum lipid phase transitions and water barrier properties. *Biochem* **26**:2382–2388 (1987).
5. D. C. Swartzendruber, P. W. Wertz, D. J. Kitko, K. C. Madison, D. T. Downing. Molecular models of the intercellular lamellae in mammalian stratum corneum. *J Invest Dermatol.* **92**:251–257 (1989).
6. S. Y. E. Hou, A. K. Mitra, S. H. White, G. K. Menon, R. Ghadially, P. M. Elias. Membrane structures in normal and essential fatty acid-deficient stratum corneum: characterization by ruthenium tetroxide staining and X-ray diffraction. *J Invest Dermatol.* **96**:215–223 (1991).
7. H. E. Bodde, B. Holman, F. Spies, A. Weerheim, J. Kempenaar, M. Mammaas, M. Ponec. Freeze-fracture electron microscopy of *in vitro* reconstructed human epidermis. *J Invest Dermatol* **95**:108–116 (1990).
8. G. F. Odland, K. Holbrook: The lamellar granules of epidermis. *Curr Probl Derm* **9**:29–49 (1981).
9. L. Landmann. The epidermal permeability barrier. Comparison between in vivo and in vitro lipid structures. *Eur J Cell Biol* **33**:258–264 (1984).
10. L. Landmann. Epidermal permeability barrier: transformation of lamellar granules-disks into intercellular sheets by a membrane-fusion process, a freeze-fracture study. *J Invest Dermatol* **87**:202–209 (1986).
11. P. W. Wertz, D. C. Swartzendruber, D. J. Kitko, K. C. Madison, D. T. Downing. The role of the corneocyte lipids envelopes in cohesion of stratum corneum. *J Invest Dermatol* **93**:169–172 (1989).
12. G. K. Menon, P. M. Elias. Ultrastructure localization of calcium in psoriatic and normal human epidermis. *Arch Dermatol* **127**:57–63 (1991).
13. B. Melnik, J. Hollmann, U. Hofmann, M. S. Yuh, G. Plewig. Lipid composition of outer stratum corneum and nails in atopic and control subjects. *Arch Dermatol Res* **282**:549–551 (1990).
14. K. R. Feingold, B. E. Brown, S. R. Lear, A. H. Moser, P. M. Elias. Effect of essential fatty acid deficiency on cutaneous sterol synthesis. *J Invest Dermatol* **87**:588–591 (1986).
15. C. Schmidt, N. S. Pennys, V. A. Ziboh, I. Kiem, J. Schlossberg. Cholesterol and cholesterol ester content in normal and pathological scale. *J Invest Dermatol* **68**:206–209 (1977).
16. K. K. Mustakallio, U. Kiistala, H. J. Piha, E. Nieminen. Epidermal lipids in Besnier's prurigo (atopic dermatitis). *Ann Med Exp Fenn* **45**:323–325 (1967).
17. P. J. Hartop, C. F. Allenby, C. Prottey. Comparison of barrier function and lipids in psoriasis and essential fatty acid-deficient rats. *Clin Exptal Dermatol* **3**:259–267 (1978).
18. Y. Werner, M. Linberg. Transepidermal water loss in dry and clinically normal skin in patients with atopic dermatitis. *Acta Derm Venereol* **65**:102–105 (1985).
19. A. W. Fulmer, G. J. Kramer. Stratum corneum lipid abnormalities in surfactant-induced dry scaly skin. *J Invest Dermatol* **86**:598–602 (1986).
20. G. Grubauer, K. R. Feingold, R. M. Harris, P. M. Elias. Lipid content and lipid type as determinants of the epidermal permeability barrier. *J Lipid Res* **30**:89–96 (1989).
21. G. M. Golden, D. B. Guzek, R. R. Harris, J. E. McKie, R. O. Potts. Lipid thermotropic transitions in human stratum corneum. *J Invest Dermatol* **86**:255–259 (1986).
22. B. F. van Duzee. Thermal analysis of human stratum corneum. *J Invest Dermatol* **65**:404–408 (1975).
23. J. Umemura, D. G. Cameron, H. H. Mantsch. A Fourier transform infrared spectroscopic study of the molecular interaction of cholesterol with DPPC. *Biochim Biophys Acta* **602**:32–44 (1980).
24. J. A. Bouwstra, G. S. Gooris, J. A. van der Spek, W. Bras. Structural investigations of human stratum corneum by small-angle x-ray scattering. *J Invest Dermatol* **97**:1005–1012 (1991).
25. D. S. Johnston, D. Chapman. A calorimetric study of the thermotropic behavior of mixtures of brain cerebrosides with other brain lipids. *Biochimica et Biophysica Acta* **939**:603–614 (1988).
26. J. B. Finean, W. Hutchinson. X-ray diffraction studies of lipid phase transitions in cholesterol-rich membranes at sub-zero temperatures. *Chemistry and Physics of Lipids* **46**:63–71 (1988).
27. S. H. White, D. Mirejovsky, G. I. King. Structure of lamellar lipid domains and corneocyte envelopes of murine stratum corneum. An X-ray diffraction study. *Biochem* **27**:3725–3732 (1988).
28. G. Grubauer, K. R. Feingold, PM Elias. Relationship of epidermal lipid synthesis to cutaneous barrier function. *J Lipid Res* **28**:746–752 (1987).
29. K. R. Feingold, M. Mao-Qiang, E. Proksch, G. K. Menon, B. E. Brown, P. M. Elias. The lovastatin-treated rodent: a new model of barrier disruption and epidermal hyperplasia. *J Invest Dermatol* **96**:201–209 (1991).
30. E. Proksch, K. R. Feingold, M. Mao-Qiang, P. M. Elias. Barrier function regulates epidermal DNA synthesis. *J Clin Invest* **87**:1668–1673 (1991).