ELSEVIER

Biochimica et Biophysica Acta 1372 (1998) 135–140

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

In vitro reconstitution of stratum corneum lipid lamellae

Deena Kuempel, Donald C. Swartzendruber, Christopher A. Squier, Philip W. Wertz *

Dows Institute, University of Iowa, Iowa City, IA, USA

Received 18 December 1997; accepted 16 March 1998

Abstract

In the final stages of differentiation in the epidermis of terrestrial mammals, lipids are extruded into the intercellular spaces. The initially extruded lipid becomes transformed into broad, multilamellar sheets that are found in the intercellular spaces throughout the stratum corneum. These lamellae display an unusual alternating broad–narrow–broad pattern of lucent bands as revealed by transmission electron microscopy (TEM). This arrangement results in two periodicities that can be measured from electron micrographs and are also evident in X-ray diffraction—5 nm (broad) and 13 nm (broad–narrow–broad). The goal of the present study was to reconstitute these lamellae in vitro. Porcine stratum corneum lipids were applied to Millipore filters. The disks were placed in water and heated to 80°C for 1 h. After cooling, the disks were stored over desiccant. At each stage, the disks were prepared for TEM. TEM revealed that the application of the lipid solutions onto the disks resulted to deposition of mostly amorphous material. Heating in water resulted in the formation of many lamellae. The width of the lamellae was uniform and in the range of 5 to 6 nm with no broad–narrow–broad pattern; however, after storage under desiccating conditions, the broad–narrow–broad pattern was reproduced.

Keywords: Stratum corneum; Lipid; Barrier; Membrane; Ceramide; Epidermis

1. Introduction

The outermost portion of the epidermis of mammalian skin, the stratum corneum, provides a protective barrier that limits the penetration of topical contaminants and prevents desiccation of the underlying tissue [1]. Physically, the stratum corneum consists of an array of flat, polygonal, keratin-filled cells embedded in a matrix of lamellar lipids. Materials that penetrate through the stratum corneum do so by passive diffusion through the intercellular spaces. Therefore, it is the lamellar lipid matrix that determines the quality of the barrier.

The lipids found in epidermal stratum corneum are unusual in composition in that they consist mainly of ceramides, cholesterol and saturated fatty acids [1,2]. Unlike most other biological membranes, the intercellular lamellae contain no phospholipids.

In 1973, Breathnach et al. [3], using freeze fracture electron microscopy, demonstrated that multiple lipid lamellae are present in the intercellular spaces of epidermal stratum corneum. However, these intercellular lamellae were not visualized in transmission electron micrographs. It is now recognized that the paucity of double bonds and functional groups on the lipids present in stratum corneum does not permit

* Corresponding author. N450 DSB, Dows Institute, University of Iowa, Iowa City, IA 52242, USA. Fax: +1-319-335-8895; E-mail: philip-wertz@uiowa.edu

0005-2736/98/$19.00
PII S0005-2736(98)00053-4

sufficient reaction with the osmium tetroxide that is routinely used in the preparation of samples for TEM [4]. When, in 1987, the stronger oxidizing agent, ruthenium tetroxide, was substituted for osmium tetroxide, the intercellular lamellae were clearly revealed [5]. Since the introduction of the ruthenium tetroxide method, numerous studies have been performed with this reagent to examine the organization of stratum corneum lipids [6–8].

Although the number of lamellae and details of the lamellar arrangement varies throughout the intercellular spaces of the stratum corneum, the most frequent lamellar arrangements contain one or more sets of broad–narrow–broad electron lucent bands [9]. In porcine stratum corneum, the most abundant arrangement is a 6-banded broad–narrow–broad–broad–narrow–broad pattern [9]. Multiple lamellae are found in the intercellular spaces at all levels within the stratum corneum, even after desquamation [10].

The purpose of the present study was to determine the conditions under which the broad–narrow–broad pattern can be reconstituted from stratum corneum lipids in vitro. The ability to reconstitute this lipid lamellar system in vitro could provide a useful model for physical studies of the stratum corneum membrane organization and could possibly be useful for in vitro studies of transdermal drug delivery.

2. Materials and methods

2.1. Preparation of stratum corneum lipid-loaded disks

The epidermis was separated from an intact pig carcass [2]. Application of an aluminum cylinder heated to 65°C for 1 min permitted separation of the epidermis from the underlying connective tissue. To isolate the stratum corneum, the epidermis was spread with the outer surface up on filter paper pads saturated with 0.5% trypsin [2] in 20 mM phosphate buffered saline (PBS, pH 7.4, 0.15 M NaCl). After incubation at 4°C overnight, the stratum corneum was extensively rinsed with distilled water. To remove final traces of noncornified cells, the stratum corneum was treated with fresh 0.5% trypsin in PBS for 2 h at 37°C followed by additional rinsing in distilled water.

Pieces of stratum corneum were frozen and dried by lyophilization. The dried tissue was extracted successively at 2-h intervals with chloroform:methanol, 2:1, 1:1 and 1:2 [2]. The combined extracts from each piece of tissue were dried under a gentle stream of nitrogen and redissolved in 5 ml chloroform:methanol, 2:1. A 1-ml portion of 2 M potassium chloride solution was added to this solution, and after vigorous shaking, the mixture was centrifuged at $1000 \times g$ for 5 min. The lower phase was transferred to a glass screw cap tube and dried under nitrogen. The lipid was dissolved in *n*-hexane:isopropanol, 3:2, at a concentration of 2 mg/ml.

Millipore filter disks (1.5 cm in diameter, circular, 0.025 μm pore size; Millipore, Bedford, MA, USA) were soaked for 5 min in hexane:isopropanol, 3:2, after which they were allowed to thoroughly air dry. Lipid solution was slowly applied to the dull side of the filter disks using a 100-μl Hamilton syringe while allowing solvent to evaporate after each 5- to 10-μl application. A total of 150 μg of lipid was applied per disk. The disks were suspended in a beaker of distilled water and heated in an oven at 80°. After cooling to room temperature, some disks were placed in a closed chamber over silica gel desiccant and stored for 2 days. After desiccation, some disks were resuspended in water for 1 h. Some of these were kept at room temperature while others were reheated at 80°.

2.2. Electron microscopy [5]

The filter disks were fixed for 2 h in 2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate at 4°C. They were then post-fixed for 1 h in 0.2% ruthenium tetroxide in 0.1 M cacodylate buffer at pH 6.8. The specimens were then dehydrated in a series of ethanol solutions (30, 50, 70, 95 and 100%) before embedding in Spurr's resin. Ultrathin sections were cut and collected on carbon-stabilized Formvar-coated grids. Sections were stained with uranyl acetate and lead citrate or, in some cases, with lead citrate alone prior to examination in a Zeiss EM 10.

3. Results

TEM of untreated Millipore filters reveals a grainy, moderately electron dense matrix with electron lucent

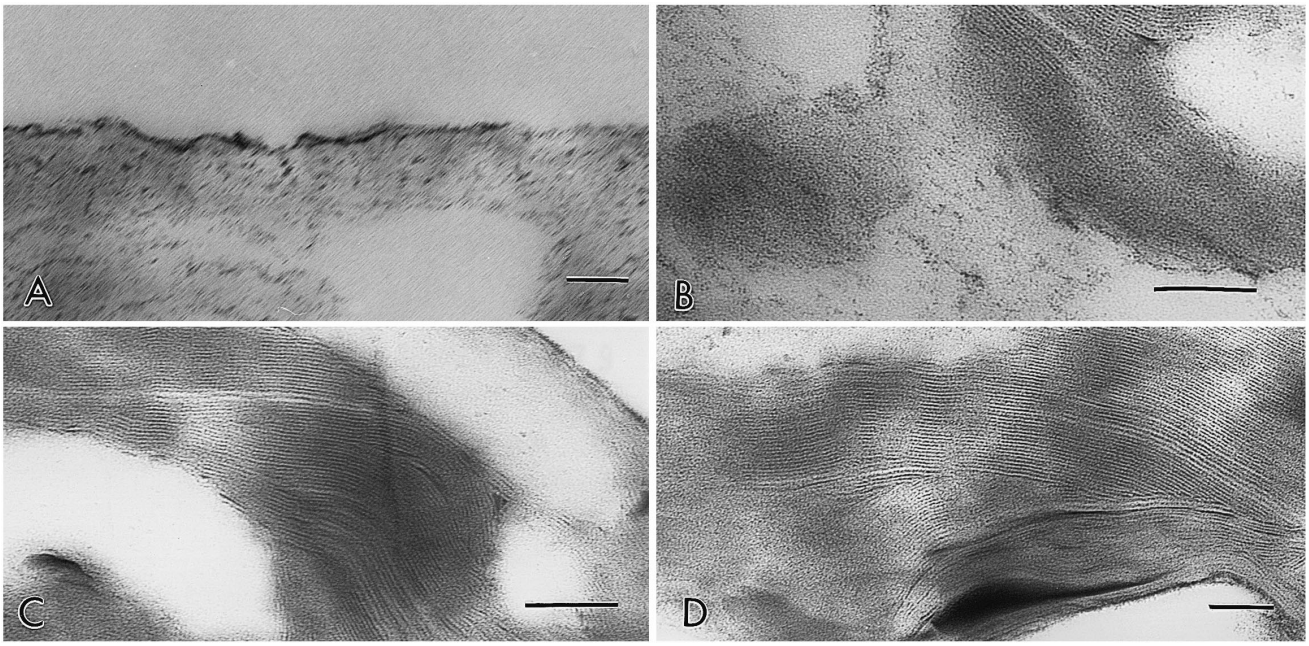

Fig. 1. TEM of stratum corneum lipids within the pores of Millipore filter disks. (a) Millipore filter without lipid. (b) Stratum corneum lipids applied from organic solvent. (c) After application of lipids, the disks were heated to 80° in water and then cooled to room temperature. (d) After heating in water, the disk was stored over desiccant. All bars represent 100 nm.

pores (Fig. 1a). When lipid is simply dried on the filter, one sees a mixture of lamellae and amorphous material within the pores of the disk (Fig. 1b); however, if the disk is heated in the presence of water to allow chain melting and hydration, uniform lamellae without amorphous material predominate (Fig. 1c). Presumably, hydration provides a driving force for the formation of bilayers. It is noteworthy that the lamellae in Fig. 1b and c are uniform in thickness and average about 5 nm. When the hydrated disks are subsequently dried over desiccant, the broad–narrow–broad lucent band pattern seen in vivo (Fig. 2) is regenerated (Fig. 1d). Once this pattern has formed, immersion of the disk in water at room temperature causes no change, but heat and hydration can cause reversion to lamellae of uniform thickness (not shown). Fig. 2 illustrates the lamellar arrangement seen in the intercellular spaces of human stratum

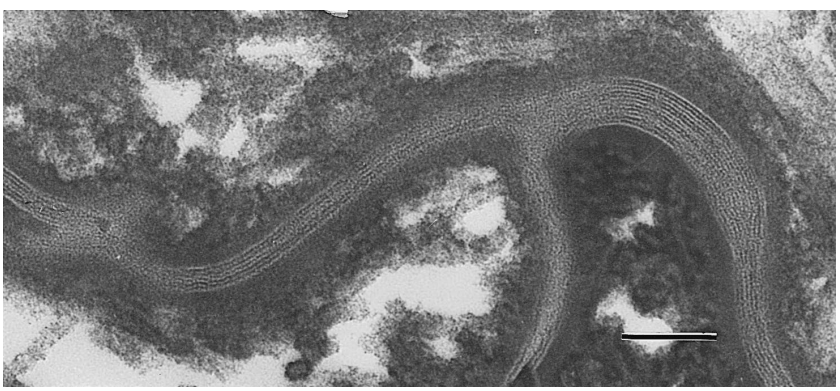

Fig. 2. Intercellular lamellae in human stratum corneum. The bar represents 100 nm.

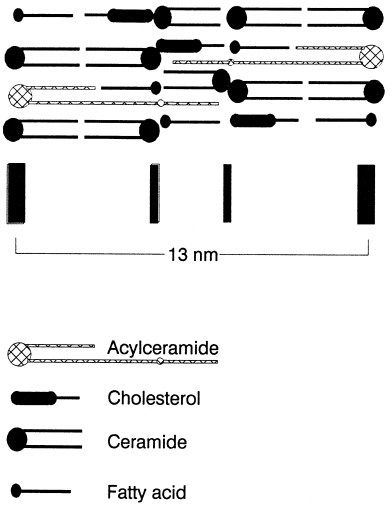

Fig. 3. A possible molecular arrangement to account for the broad–narrow–broad pattern. Note that the acylceramide spans a broad lamella while the linoleate tail inserts into a narrow lamella. The molar ratio of fatty acid to cholesterol to ceramide is approximately 1:2:3 [2]. A specific role is proposed for the acylceramide, but the locations of the other lipids in this model are arbitrary and not intended to imply any specific function.

corneum, and Fig. 3 depicts a possible molecular arrangement that could account for the observed broad–narrow–broad pattern.

4. Discussion

Madison et al. [5] achieved the first visualization of the broad-narrow-broad arrangement of the stratum corneum intercellular lipid lamellae. It was recognized at this time that the broad lamellae in the stratum corneum, as well as the precursor lamellae in the lamellar granules, have the dimensions of a typical lipid bilayer, approximately 5 nm, whereas, the broad–narrow–broad units were 13 nm wide.

Subsequent X-ray diffraction studies of the stratum corneum also revealed major periodicities of approximately 5–6 nm and 13 nm, and these periodicities were attributed to the lamellar organization of the intercellular lipids [6,11–14]. In 1988, White et al. [11] suggested that the 13 nm repeat unit represented a lipid bilayer–protein complex. This suggestion was based, in part, on the observation that the extracted lipids dispersed in excess water did not give a simple lamellar diffraction pattern. However, more recent X-ray diffraction studies have shown that both the 5–6 nm and 13 nm repeats can be reproduced with stratum corneum lipids in the absence of any protein [14]. The present results confirm that, under the right conditions, extracted stratum corneum lipids can be reconstituted into the broad–narrow–broad arrangements. These actually consist of paired bilayers with intervening narrow, interdigitated layers. The 13 nm periodicity corresponds to the distance from the center of one pair of bilayers to the center of the next pair of bilayers.

Of the ceramides present in the stratum corneum, the least polar ceramide is structurally unusual [2]. This acylceramide consists of 30- through 34-carbon long ω-hydroxyacids amide-linked to sphingosine and has linoleic acid ester-linked to the ω-hydroxyl group. Several studies employing X-ray diffraction have demonstrated a requirement for this unusual ceramide for the in vitro formation of the 13 nm periodicity [15,16]. Also, it has recently been shown that in oral stratum corneum, where the proportion of the linoleate-containing ceramide is markedly lower than in epidermal stratum corneum, the broad–narrow–broad pattern is never seen by TEM [2]. Given the dimensions of the acylceramide, it has been proposed that the long ω-hydroxyacyl portion of this unusual molecule spans one broad layer while the linoleate tail inserts into a narrow layer [16,17].

It is noteworthy that the present study requires the application of ruthenium tetroxide fixation. The principal lipids from the stratum corneum are ceramides, cholesterol and saturated fatty acids, and in a previous study [4], it was demonstrated that these lipids do not react appreciably with osmium tetroxide, which is routinely used in preparation of specimens for TEM. Accordingly, the lamellae formed from the stratum corneum lipids can be visualized after fixation with the chemically more reactive ruthenium tetroxide but are not evident in the samples prepared using osmium tetroxide.

The present results demonstrate that hydration and heat facilitate the formation of lipid lamellae. It is generally thought that the interaction of dispersed bipolar lipids with water will form bilayers because this allows the hydrophobic aliphatic chains to avoid unfavorable interactions with the aqueous environment while, at the same time, allowing maximum hydrogen bonding between the lipid polar head groups

and water. It seems most likely that lipid vesicles form initially, and that these vesicles subsequently fuse to form the broad lamellar structures seen in the pores of the filter disks. Interestingly, desiccation was required to produce the broad–narrow–broad lamellar arrangement. Hydration followed by dehydration mimics the change in water availability that would be encountered in vivo in passing from the aqueous environment of the granular cells into the relatively dry stratum corneum. The elevated temperature used during the hydration step is not physiological but is necessary to melt the aliphatic chains to permit bilayer formation. In vivo, the precursors to the stratum corneum lamellae already exist in bilayer form.

The proposition that the acylceramide spans a broad lamella while the linoleate tail inserts into a narrow lamella would result in all of the lipid layers being effectively cross-linked. In this regard, the narrow lucent bands have been likened to 'molecular zippers' [17]. This cross-linking could explain the stability of the broad–narrow–broad pattern upon rehydration at room temperature as well as the remarkable earlier observation that the X-ray diffraction pattern of the stratum corneum is insensitive to the degree of hydration [18]. The water molecules cannot intercalate between the lipid layers because they are zipped together. However, if heated to permit lipid chain melting in the presence of water, the formation of the cross-linked broad–narrow–broad arrangement is reversed. Although the X-ray diffraction results [15,16] support the supra-bilayer role proposed for the acylceramide, there is presently no evidence bearing on the location or roles of the other lipids within the intercellular spaces of the stratum corneum.

The preparation of standardized disks containing known amounts of stratum corneum lipid of known composition may provide a useful standardized substrate for use in in vitro studies pertaining to transdermal drug delivery. Preliminary studies (not shown) have demonstrated that the lipid-loaded disks are more permeable to water than the skin, a fact that is thought to reflect differences in lipid content and overall organization of the porous disks compared to the stratum corneum. Nevertheless, the lipid-loaded disks do provide a significant barrier, and this is related to the amount of lipid applied. In going from 100 to 300 μg of applied lipid, there is little improvement in the barrier properties of the disk, and when larger amounts are applied, the additional lipid noticeably builds up on the outside of the disk. Approximately 150 μg of lipid, as used in the present investigation, appears to be an optimum amount. Both the relative permeability to different penetrants and the effects of different agents on permeability and membrane organization could be studied in this model system. These possibilities remain to be tested.

Acknowledgements

Supported in part by grants from the National Institutes of Health (RO1 DE1 1273 and RO1 DE1 0516).

References

[1] P.W. Wertz, A.J. Hoogstraate, C.A. Squier, Biochemical basis of the permeability barrier in skin and oral mucosa, in: M.J. Rathbone (Ed.), Oral Mucosal Drug Delivery, Marcel Dekker, New York, 1996, pp. 27–49.

[2] S. Law, P.W. Wertz, D.C. Swartzendruber, C.A. Squier, Regional variation in content, composition and organization of porcine epithelial barrier lipids revealed by thin-layer chromatography and transmission electron microscopy, Arch. Oral Biol. 40 (1995) 1085–1091.

[3] A.S. Breathnach, T. Goodman, C. Stolinski, M. Gross, Freeze fracture replication of cells of stratum corneum of human epidermis, J. Anat. 114 (1973) 65–81.

[4] D.C. Swartzendruber, I.H. Burnett, P.W. Wertz, K.C. Madison, C.A. Squier, Osmium tetroxide and ruthenium tetroxide are complementary reagents for the preparation of epidermal samples for transmission electron microscopy, J. Invest. Dermatol. 104 (1995) 417–420.

[5] K.C. Madison, D.C. Swartzendruber, P.W. Wertz, D.T. Downing, Presence of intact intercellular lipid lamellae in the upper layers of the stratum corneum, J. Invest. Dermatol. 88 (1987) 714–718.

[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 (1991) 215–223.

[7] R. Ghadially, L. Halkier-Sorensen, P.M. Elias, Effects of petrolatum on stratum corneum structure and function, J. Am. Acad. Dermatol. 26 (1992) 387–396.

[8] M. Fartasch, I.D. Bassukas, T.L. Diepgen, Disturbed extruding mechanism of lamellar bodies in dry non-exematous skin in atopics, Br. J. Dermatol. 127 (1992) 221–227.

[9] D.C. Swartzendruber, A. Manganaro, K.C. Madison, M. Kremer, P.W. Wertz, C.A. Squier, Organization of the

intercellular spaces of porcine epidermal and palatal stratum corneum: a quantitative study employing ruthenium tetroxide, Cell Tissue Res. 279 (1995) 271–276.
[10] P.W. Wertz, D.C. Swartzendruber, K.C. Madison, D.T. Downing, Composition and morphology of epidermal cyst lipids, J. Invest. Dermatol. 89 (1987) 419–425.
[11] 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, Biochemistry 27 (1988) 3725–3732.
[12] J.A. Bouwstra, M.A. de Vries, G.S. Gooris, W. Bras, J. Brussee, M. Ponec, Thermodynamic and structural aspects of the skin barrier, J. Control. Release 16 (1991) 209–220.
[13] J.A. Bouwstra, G.S. Gooris, J.A. van der Spek, S. Lavrijsen, W. Bras, The lipid and protein structure of mouse stratum corneum: a wide and small angle diffraction study, Biochim. Biophys. Acta 1212 (1994) 183–192.
[14] J.A. Bouwstra, G.S. Gooris, K. Cheng, A. Weerheim, W. Bras, M. Ponec, Phase behavior of isolated skin lipids, J. Lipid Res. 37 (1996) 999–1011.
[15] T.J. McIntosh, M.E. Stewart, D.T. Downing, X-ray diffraction analysis of isolated skin lipids: reconstitution of intercellular lipid domains, Biochemistry 35 (1996) 3649–3653.
[16] J.A. Bouwstra, G.S. Gooris, F.E.R. Dubbelaar, A.M. Weerheim, A.P. Ijzerman, M. Ponec, The role of ceramide 1 in the molecular organization of the stratum corneum lipids, J. Lipid Res., 1997, in press.
[17] P.W. Wertz, Integral lipids of hair and stratum corneum, in: H. Zahn, P. Jolles (Eds.), Hair: Biology and Structure, Birkhauser Verlag, Basel, 1995, pp. 227–238.
[18] J.A. Bouwstra, M.A. Salomons-de Vries, J.A. van der Spek, W. Bras, Structure of human stratum corneum as a function of temperature and hydration: a wide-angle X-ray diffraction study, Int. J. Pharm. 84 (1992) 205–216.