A Cryotransmission Electron Microscopy Study of Skin Barrier Formation

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Direct visualization of the skin barrier formation process by cryotransmission electron microscopy of vitreous epidermal sections has been performed. The results obtained differ in many aspects from those of classical chemical fixation electron microscopy. Here we show that (i) a new "organelle or branched tubular structure" containing nonlamellar or partly lamellar material, and closely corresponding in numbers, dimensions, and localization to lamellar bodies of classical chemical fixation electron micrographs, and (ii) a new "ribosome complex-like structure", not preserved in classical electron micrographs, are omnipresent at apparent active sites of skin barrier formation. Evidence that skin barrier formation may not take place via extensive membrane fusion involving discrete lamellar bodies, but rather by a morphologically continuous membrane folding process are: (i) the often clearly nonlamellar content of structures corresponding to lamellar bodies with concomitant visualization of multilamellar membrane structures of the intercellular space; (ii) the "multifolded" appearance of the lipid matrix of the intercellular space; and (iii) the identification of extended "intracellular" multilamellar continuous structures with an optical density profile closely corresponding to that of the lipid matrix of the intercellular space. Based on the cryo-electron microscopic data presented in this study we propose that a membrane transition from cubic-like to multilamellar may take place already inside the "tubuloreticular cisternal membrane system" of upper granular cells recently reported by Elias *et al.*

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The pronounced gradients (e.g., in water chemical potential) present over the stratum corneum indicate that the stacked lamellar lipid matrix of the stratum corneum intercellular space, like most other biologic systems, may not essentially be an equilibrium structure, but rather stabilized by secondary, or higher, minimum energy order steady states (Peacocke, 1983). Consequently, the way in which this multilamellar membrane structure, representing the skin barrier, has been formed may have profound implications for its structural organization and physical characteristics (Norlén, 2002).

A fundamental problem for skin barrier research is that it is not known what artifacts are introduced during standard sample storage and sample preparation procedures for, for example, X-ray diffraction and electron microscopy. Separation of epidermis, with or without heat, trypsinization, dehydration in a desiccation chamber, storage in a freezer as well as chemical fixation, solvent exposure, dehydration, and staining during sample preparation for electron microscopy, etc., are procedures that may have profound effects on the structural organization of lipid membranes and other constituents of the epidermis. One potential way, however, to obtain topologic as well as other morphologic skin data largely unaffected by preparation induced artifacts and the forced equilibrium (or perturbed nonequilibrium) conditions of the *in vitro* situation, may be by the use of cryotransmission electron microscopy of vitrified epidermal sections.

In the classical model for skin barrier formation presented by Landmann (1986), so-called "lamellar bodies", containing in their turn "lamellar disks", were proposed to bud off from the *trans*-Golgi network and diffuse upwards towards the plasma membrane of the differentiating stratum granulosum cells. After fusion of the limiting membrane of the lamellar bodies with the cell envelope of the stratum granulosum transition cell the lamellar disks were proposed to be discharged into the intercellular space where they would merge into intercellular lamellar sheets via a second fusion process.

Fusion processes are, however, energetically unfavorable and imply a topology change that does not preserve membrane continuity, which may be essential both for barrier capacity and for stratum granulosum tissue water compartmentalization (i.e., homeostasis) during skin barrier formation. Further, targeted vesicular diffusion is time consuming and massive, coordinated vesicular fusion poses, from a biophysical point of view, a formidable problem of process control.

It has therefore been proposed that skin barrier formation may take place as a morphologically continuous "flattening" or "unfolding" of a three-dimensional cubic-like membrane structure into the flat multilayered two-dimensional lipid structure of the stratum corneum intercellular space representing the skin barrier (Norlén, 2001). This model, the "membrane folding model", does not, in contrast to the "Landmann model", involve any membrane topology change.

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Abbreviations: OD, optical density

In this study cryotransmission electron microscopy of thin vitrified epidermal sections from human volar forearm skin was used to study qualitatively the morphology of apparent active sites of skin barrier formation, at the interzone between stratum granulosum and stratum corneum.

MATERIALS AND METHODS

Tissue preparation Skin biopsies were taken during the fall/winter of 2001/2002 from the mid portion of the left volar forearm of four healthy (as judged by dermatologic examination) caucasian male subjects (24–34 y old) that had no history of dermatologic disease. The skin area used had not been exposed to any detergents, treatments, or skin care products for 1 mo prior to experimentation. The samples were cut with a double-edged razorblade into 1×1 mm^2 large pieces with a thickness of approximately 50–100 μm, and subsequently placed in the cavity (diameter 2 mm; depth 0.1 mm) of a cylindrical aluminum platelet (diameter 3 mm; thickness 0.5 mm) and covered by a second matching flat aluminum platelet. The cavity space not occupied by the sample was filled with 1-hexadecane (Fluka, Buchs, Switzerland). Human studies were performed according to the Declaration of Helsinki Principles and were approved by the Institutional Review Board. There was informed consent of human volunteers.

High-pressure freezing The high-pressure freezer HPM 010 (Baltec, Liechtenstein), which reaches a pressure of 1700 bar within 15 ms, was used. The high pressure was built up at a controlled temperature by the use of a defined volume of thermostatized ethanol that hit the sample before the pressurized liquid nitrogen and thus ensured a stable correlation between rise in pressure and drop in temperature (Moor, 1987).

Cryosectioning For a general description of cryo-electron microscopy of vitreous specimens Dubochet *et al* (1988).

Small pieces of the high-pressure frozen epidermal forearm samples were glued to aluminum pins in a FCS cryo-chamber of an Ultracut S microtome (Reichert FCS, Leica, Vienna). The vitreous samples were trimmed with a trimming diamond blade (Diatome, Biel, Switzerland) and cryosectioned at 110 K with a nominal section thickness of 50 nm using a 45° diamond knife (Diatome) with a clearance angle of 6°. Cutting speed was set to 0.6 mm per second. The sections were transferred to carbon-film covered copper grids with 400 and 1500 mesh, respectively, using an eyelash glued to a wooden stick. Subsequently, they were pressed with a stamping tool and stored in liquid nitrogen. A facemask was used all through the section transfer procedure to minimize ice-crystal contamination.

Cryo-electron microscopy The copper grids with the mechanically attached vitreous epidermal sections were transferred to a Gatan cryoholder (Gatan Inc., Warrendale, PA) at 110 K and inserted in a Philips CM12 cryoelectron microscope (Philips, Eindhoven, the Netherlands) equipped with a tungsten filament. The accelerating voltage was 80 kV, objective aperture 50 μm and camera length 370 mm. The applied electron dose was about 400 e per nm^2 at an electronic magnification ranging from 3000 to 13,000. Some regions, however, were more severely irradiated due to prolonged observation. Images were recorded digitally with the use of a Gatan CCD camera (Gatan, Inc.). In total, more than 200 vitreous sections were examined.

Defocus and resolution Defocus and image resolution was calculated using the EMtool® software (Ludtke, 1996).

Contrast measurements The optical density (OD) of micrographs to be compared, were calibrated with a standard negative of known OD. Contrast between adjacent regions with different OD (OD_1 and OD_2) was defined as $[OD_1–OD_2]/[(OD_1 + OD_2)/2]$. OD was measured using a transmission densitometer (Melico/Photolog, Medical & Electrical Instrumentation Ltd, London, UK)

Section thickness and mass loss determination The thickness of the sections was determined from their contrast on the micrographs, on the basis of the following relation (Dubochet *et al*, 1983):

$$\rho t = \Lambda \ln(ODh/OD)$$

where OD represents the OD of the different biologic structures of the sample, ODh represents the OD of a hole in the section, ρ is the density, t the section thickness, and Λ the mean free mass thickness of the electrons through the specimen. For our working conditions Λ corresponds to 100 nm water.

RESULTS

It is emphasized that the results presented below are purely qualitative and that interindividual differences are likely to play significant roles. In several micrographs presented here defocus and/or electron dose was not optimal.

Epidermal cells situated above the uppermost nucleated granular cell and below the lowermost cornified cell, are hereafter termed "transition cells". Dilated membrane-containing regions of the intercellular space situated at the interzone between stratum granulosum and stratum corneum, with associated clusters of intracellular organelles, are referred to as "apparent active sites of skin barrier formation".

Methodologic evaluation

High-pressure freezing The time elapsed between biopsy taking and vitrification of the sample was approximately 90 s (range 60–180 s, n = 36), and primarily limited by the regeneration of the high-pressure freezer between runs.

Electron diffraction The diffraction patterns of vitrified frozen hydrated human mid-volar forearm epidermis showed a first diffuse ring corresponding to 0.37 nm, typical of low-density vitreous water. This is in contrast to the diffraction pattern of hexagonal ice with characteristic reflextions corresponding to 0.39, 0.37, and 0.34 nm and the diffraction pattern of cubic ice with characteristic continuous ring corresponding to 0.37, 0.22, and 0.19 nm (Dubochet *et al*, 1988).

Section thickness, ice contamination, and cutting artifacts Ice contamination during sample handling was low and did not affect the quality of the micrographs in a significant manner.

The median thickness of the cryosections was 100 nm (range 80–110 nm, n = 20). Compression along the cutting direction was in the order of 30–60%, most easily detectable as an oval shape deformation of small and medium sized mitochondria.

Knife marks were present on all vitreous sections of cryo-fixated epidermis, but did not affect the quality of the micrographs in a significant manner.

Ultrastructural evaluation

The transition cell Apparent active sites of skin barrier formation appeared distributed all around the periphery of the epidermal transition cells, and to a roughly equal extent at the apical and caudal parts (**Fig 1**, *open white arrows*). This is in accordance with what has been reported for chemically fixed epidermis (Fartasch *et al*, 1993).

Active sites of skin barrier formation Two cellular structures were distinguished at active sites of skin barrier formation. The first was a new "organelle or branched tubular structure" (**Fig 2*a***, *open white arrows*), closely corresponding in numbers, dimensions, and localization to lamellar bodies of classical chemical fixation electron micrographs (**Fig 2*b***, MG). The second was a new "ribosome complex-like structure" (**Fig 2*a***, *open black arrows*) in apparent continuity with a multivesicular (white asterix) or multifolded (black asterix) membrane complex (*trans*-Golgi network?) of larger dimensions. The new "ribosome complex-like structure" often shared the space between the densely packed tonofilament bundles (TF in **Fig 2**) with the new "organelle or branched tubular structure", and gave the impression of being in possible continuity with the tonofilament bundles. It may be noted that this new "ribosome complex-like structure" is not preserved in classical chemical fixation electron micrographs (cf. **Fig 2*b***, empty ribosome-studded space).

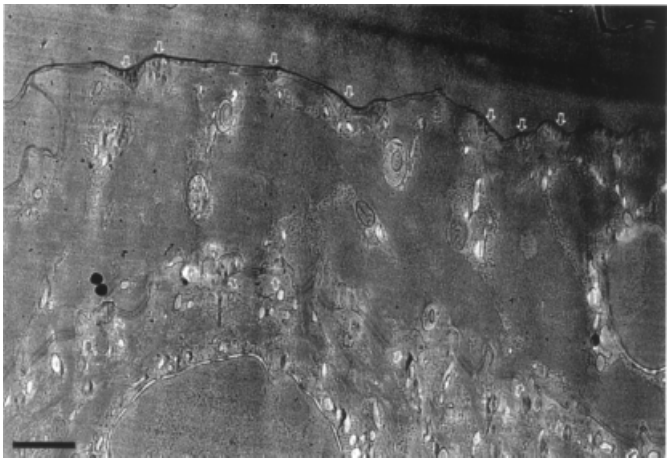

Figure 1. Apparent active sites of skin barrier formation (*open white arrows*) are distributed all around the border of the stratum granulosum/stratum corneum transition cell. Cryotransmission electron micrograph of the stratum granulosum/stratum corneum interface. Defocus: –6 μm. Optical resolution: 5 nm. *Scale bar* 500 nm.

Further, a striking difference in the cellular volume occupied by tonofilament bundles was apparent between the cryo-fixed (**Fig 2*a***, transition cell) and chemically fixed (**Fig 2*b***, transition cell) epidermal cells. There was no cytoplasmic or intracellular space devoid of densely packed organic material present in any of the cryo-fixed epidermal samples under study (**Figs 1–6**).

New "organelle or branched tubular structure" The new "organelle or branched tubular structure" contained nonlamellar or partly lamellar material (**Fig 3**, *open white and black arrows, left and upper right inset*). It was, however, never empty or partly empty, as reported for corresponding "lamellar bodies" of classical chemical fixation electron micrographs (Brody, 1989; Madison *et al*, 1998). The content of the new "organelle or branched tubular structure" frequently showed circular or tubular inclusions with a central electron dense spot, embedded in a nonlamellar matrix (**Fig 3**, *open white arrows, left inset*). The median mean diameter of the new "organelle or branched tubular structure" was 150 nm (range 115–165 nm, n = 15) and the median mean diameter of the inclusions was 30 nm (range 25–35, n = 19).

Occasionally, the electron density pattern corresponding to the limiting membrane of the new "organelle or branched tubular structure" showed an apparent inverted S-shaped appearance (**Fig 3**, *black asterixes*), suggesting a continuity between adjacent new "organelles", and thus favoring the idea of their true morphology being that of a single "branched tubular structure". A similar OD pattern (*white asterixes*) was also found in invaginations of the transition cell membrane (*solid white arrow*), in a "multivesicular-like complex" of smaller dimensions (*solid black arrow*), and in the transition cell plasma membrane (**Fig 3**, *topmost white asterix*). This suggests a possible continuity between the limiting membrane of the new "branched tubular structure" and the transition cell plasma membrane, and, by inference, a continuity between the organic content of the new "branched tubular structure" and the intercellular space.

New "ribosome complex-like structure" A characteristic morphologic feature of the transition cell cytoplasm at apparent active sites of skin barrier formation was a seemingly continuous transition (**Fig 4**, *white asterixes*; **Fig 2*a***, *black asterix*) between the "ribosome complex-like structure" (*open black arrows*) and a multifolded membrane complex of larger dimensions (*trans*-Golgi network?) (**Fig 4**, *open white arrow*; **Fig 2*a***, *black and white asterix*).

New "continuous multilamellar structure" Occasionally, folded multilamellar continuous structures (**Fig 5**, *solid white arrow, inset*) were detected adjacent to apparent active sites of skin barrier formation. A similar multilamellar OD pattern was found in the intercellular space (**Fig 5**, *solid and open black arrows*), indicating a possible continuity between the new "continuous multilamellar structure" and the lipid matrix of the intercellular space.

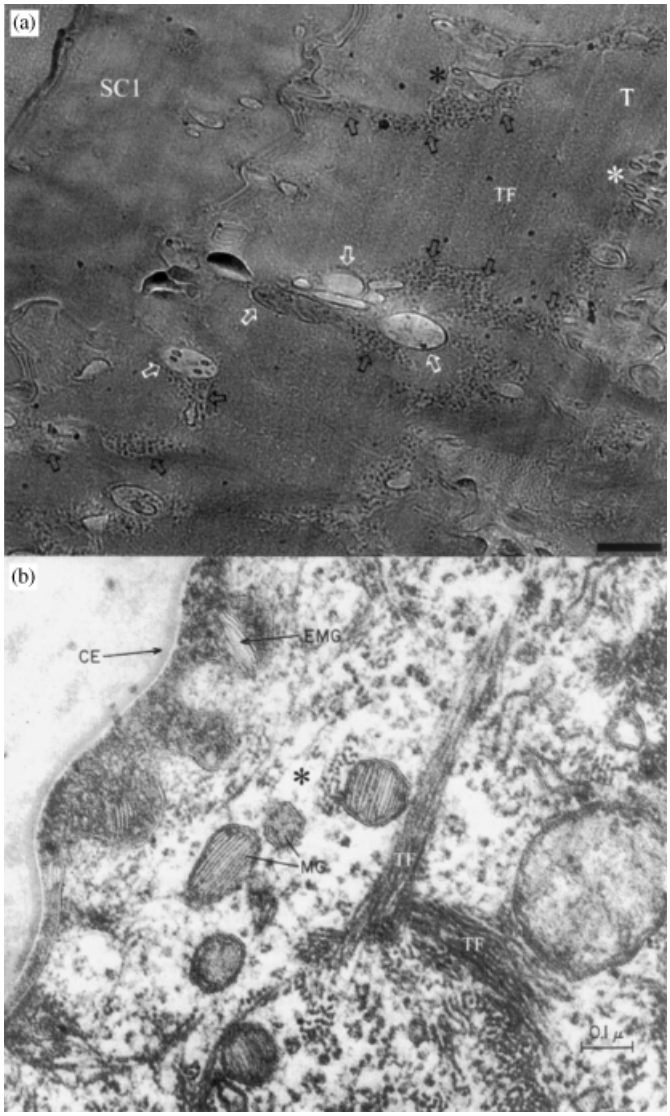

Figure 2. (*a*) A new "organelle or branched tubular structure" (*open white arrows*), corresponding to "lamellar bodies" of classical chemical fixation electron micrographs, and a new "ribosome complex-like structure" (*open black arrows*), not preserved in classical electron micrographs, were both omnipresent at apparent active sites of skin barrier formation. Cryotransmission electron micrograph of the stratum granulosum/stratum corneum interface. T: transition cell; SC1: lowermost cornified cell. *Black and white asterix*: multifolded or multivesicular membrane complex. TF: tonofilaments. Defocus: –15 μm. Optical resolution: 8 nm. *Scale bar*: 200 nm. (*b*) Classical chemical fixation electron micrographs of the stratum granulosum/stratum corneum interface characteristically shows large artifactual "empty" areas (*black asterix*) and artifactual aggregation of tonofilament structures (TF) into distinct bundles. MG: lamellar body within stratum granulosum cell; EMG: lamellar body in the intercellular space; CE: cornified envelope. With permission from the Macmillan Press Ltd (Stenn 1983, p 577).

Intercellular lipid matrix At large active sites of skin barrier formation the intercellular space showed characteristically a strongly folded multilamellar appearance (**Figure 5**, *open black arrow*).

A multilamellar OD pattern, representing stacked lipid membranes of the newly formed skin barrier, with a median repeat distance of 6.0 nm (range 5.5–6.5 nm, n = 10) was clearly visible in the first stratum corneum intercellular space (**Fig 6**).

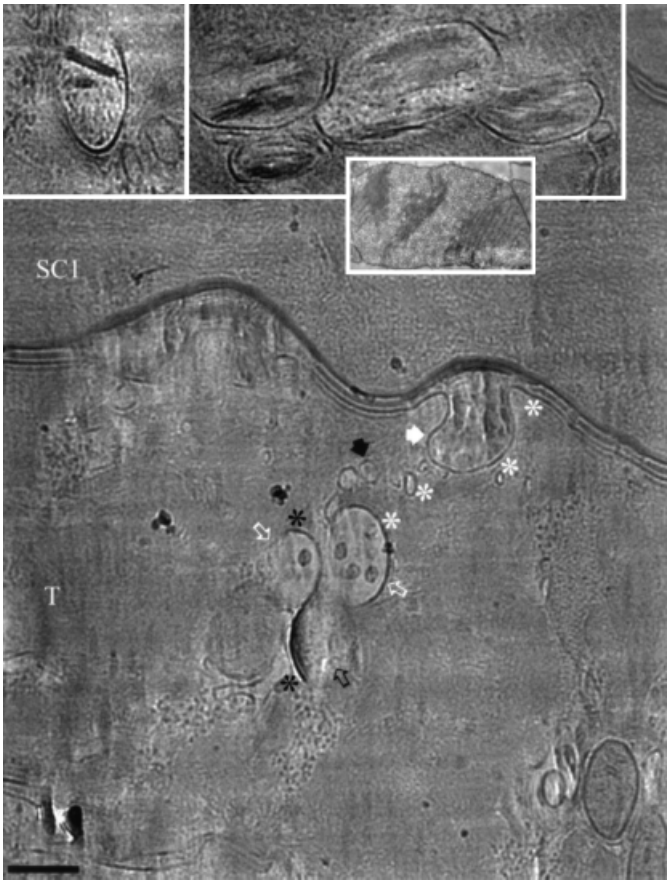

Figure 3. The new "organelles or branched tubular structures" contain granular material (*open black arrow*) with or without circular (*open white arrow*) or tubular-like (*left inset*) or multilamellar (*upper right inset*) inclusions. Cryotransmission electron micrographs of the stratum granulosum/stratum corneum interface. Note the similarities between the partly granular/lamellar content of the "organelles or branched tubular structure" (*right upper inset*) and the cubic-to-lamellar membrane transition (*right lower inset*), i.e., foldings/unfoldings between cubic (three-dimensional hyperbolic) and lamellar (two-dimensionally Euclidian) membrane structures, in a classical electron micrograph of mitochondriae of cardiac muscle cells of *Serinus canarius* (canary) (Slautterback, 1965; Landh, 1996, p 81, with permission). Cubic membranes can, in fact, sometimes be unambiguously identified and defined from two-dimensional electron micrographs through a direct template-correlation procedure (Landh, 1996, pp 67–69). T: transition cell; SC1: lowermost cornified cell; *solid black arrow*: membrane complex; *solid white arrow*: invagination of the transition cell plasma membrane. Defocus: −13 μm. Optical resolution: 7 nm. *Scale bar*: 100 nm.

Figure 4. The new "ribosome complex-like structure" is derived from a large electron lucent structure with a "multifolded" appearance. Cryotransmission electron micrograph of a new "ribosome complex-like structure", not preserved in classical electron micrographs, at the stratum granulosum/transition cell interface. Note the seemingly continuous transition (*white asterix*) between a large electron lucent structure with a "multifolded" appearance (*trans*-Golgi complex?) (*open white arrow*) and the new "ribosome complex-like structure" (*open black arrows*), and its resemblance to the cubic-to-cubic membrane transition in a classical electron micrograph of a single and coherent endoplasmic reticulum (ER) of differentiating sieve elements (*inset, upper half*: di-gyroid ER with a lattice parameter of 140 nm; *inset, lower half*: mono-gyroid ER with a lattice parameter of 50 nm) (with permission: Behnke, 1968; Landh, 1996). SG: uppermost nucleated stratum granulosum cell; T: transition cell; D: desmosome; *black asterix*: invagination of the plasma membrane. Defocus: −13 μm. Optical resolution: 7 nm. *Scale bar*: 100 nm.

DISCUSSION

Cryotransmission electron microscopy of vitreous sections of human epidermis may constitute a suitable tool to study the morphologic changes involved in skin barrier formation. This is not only because chemical fixation, dehydration, and staining during classical sample preparation for electron microscopy, with possible severe perturbation (if not complete destruction) of different biologic structures is altogether avoided during high-pressure cryofixation, but also because different irreversible constraints (e.g., different energy gradients) that govern epidermal ultrastructural organization (Peacocke, 1983) may partly be "frozen in time". These advantages should, however, be weighted against possible pressure-induced artifacts, which by no means can be disposed of. This is because many dynamical biologic processes, such as membrane and protein oscillations, take place in the time scale of <1 ms, whereas cryo-fixation time is in the order of 20 ms.

The most striking difference between epidermal cryo-electron micrographs (**Fig 2*a***) and classical electron micrographs (**Fig 2*b***) is the considerably more densely packed appearance of the former. This is, without doubt, mainly due to superior tissue preservation after vitrification (**Fig 2*a***). The difference is alarming, as a major part of the total biomass seems, by comparison, to be lacking in the classically fixed epidermis (**Fig 2*b***). Ironically, the absence of some biostructures gives a higher optical contrast to remaining structures, largely due to a reduction in morphologic complexity. The "biologic contrast" (i.e., the amount of actual ultrastructural information contained) is, however, strongly reduced. The dominating problem with classical electron microscopy is therefore that we here are confronted with important biologic information gaps and that we are looking at artifacts of uncertain biologic relevance.

The main problem with cryo-electron microscopy is thus not the "contrast" as one here is faced, not with a lack of, but with a plethora of biologic information. The overlapping of biostructures in the section thickness dimension is consequently more pronounced than for classical chemical fixation electron microscopy. This renders the interpretation of cryo-electron micrographs highly complex. It is, however, not unlikely that computer-assisted cryo-electron tomography may partly solve this matter in the future.

Figure 2(*a*) shows (judging from over 200 epidermal sections examined in this study) a representative image of the interzone

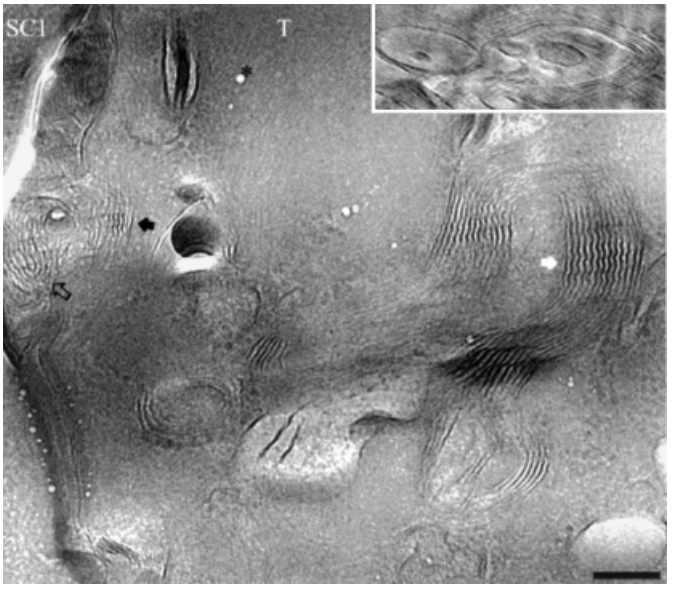

Figure 5. A new "continuous multilamellar structure", which may correspond to the "extensive tubuloreticular cisternal membrane system" reported by Elias *et al* (1998), was identified. Cryotransmission electron micrograph of a new "continuous multilamellar structure" close to the transition cell/stratum corneum interface. Note the similar layered OD profile of a region of the intercellular space (*solid black arrow*) at an active site of skin barrier formation, and of new "continuous multilamellar structure" (*solid white arrow*). Note further the strongly folded multilamellar appearance of the intercellular space (*open black arrow*). *Inset*: cryoelectron micrograph of a similar folded multilamellar continuous structure in conjunction with a new "organelle or branched tubular structure" with circular inclusion (Fig 3, *open white arrows*). *Black asterix*: bubbling artifact; T: transition cell; SC1: lowermost cornified cell. Defocus: –9 μm. Optical resolution: 6 nm. *Scale bar*: 100 nm.

Figure 6. The stratum corneum intercellular multilamellar lipid matrix is clearly visualized by cryotransmission electron microscopy. Cryotransmission electron micrograph of the intercellular space at the stratum granulosum/stratum corneum interface. Median lamellar repeat distance 6.0 nm (range 5.5–6.5 nm, n = 10). T: transition cell; SC1: lowermost stratum corneum cell. Defocus: –5 μm. Optical resolution: 3 nm. *Scale bar*: 100 nm.

between stratum granulosum and stratum corneum of cryo-fixed vitreous human forearm epidermis. Both the new "organelle or branched tubular structure" (*open white arrows*) and the new "ribosome complex-like structure" (*open black arrows*) are present.

The new "organelle or branched tubular structure" (**Fig 3**, *open white arrows*) contained nonlamellar (*open black and white arrows, left inset*) or partly lamellar (*upper right inset*) material, but was never empty or partly empty. Tentatively, three distinct types of internal material were observed, which were: (i) only granular (*open black arrow*); (ii) granular with circular (*open white arrows*) or cylindrical (*left inset*) inclusions; and (iii) granular with lamellar inclusions (*upper right inset*). The presence of an exclusively lamellar content was not confirmed in this study, but cannot be excluded as the visualization of multilamellar structures largely depends on the orientation of the section plane.

The varied appearance of the interior matrix of this structure could either be due to the presence of several distinct types of "organelles" or be the result of extensive morphologic changes taking place within one single type of structure. From a physiologic standpoint the latter is not unlikely as the interzone between stratum granulosum and stratum corneum is characterized by significant structural reorganization. Further, corresponding structures of classical electron micrographs, the "lamellar bodies", have likewise been reported to express a plethora of appearances such as being empty or partly lamellar, or to contain "onion-like" structures, or to have a granular appearance, or to exhibit "abnormal internal contents", etc. (Brody, 1989; Madison *et al*, 1998).

A striking observation is that the content of adjacent "organelles" typically show a very similar appearance (**Fig 3**, *upper right inset*), indicating a similar stage of internal development. The internal appearance of adjacent "organelles" is indeed so similar with respect to the total variability between these "organelles", that the idea of "organelles" as being parts of a single and coherent "branched tubular structure" may be considered. The inverted S-shaped appearance of the limiting membrane of some "organelle" clusters (**Fig 3**, *black asterixes*) further strengthen the hypothesis of morphologic continuity. In fact, this view is further supported by recent classical electron microscopy data showing an "extensive intracellular tubuloreticular cisternal membrane system within the apical cytosol of the outermost stratum granulosum" composed of a widely disbursed *trans*-Golgi-like network associated with arrays of contiguous lamellar bodies and deep invaginations of the stratum granulosum/stratum corneum interface (Elias *et al*, 1998).

The partly granular/lamellar interior of some "organelles" bears morphologic resemblance to the cubic to lamellar membrane transitions in mitochondriae in avian cardiac muscle cells (**Fig 3**, *lower right inset* (Slautterback, 1965; Landh, 1996). In the "membrane folding model" (Norlén, 2001), such a transition was predicted to take place in the intercellular space between the stratum granulosum and stratum corneum. According to our data this transition may thus start already inside terminally differentiated "organelles" (i.e., the lamellar bodies of classical electron micrographs) (cf. **Fig 3**, *upper right inset*), which, in fact, is to be expected if we consider that these "organelles" may form a "branched tubular structure" in continuity with the intercellular space (above).

The new "ribosome complex-like structure" filled a large part of the keratinocyte cytoplasmic space that was not occupied by tonofilament bundles, and often appeared adjacent to the cell periphery at apparent active sites of skin barrier formation (**Figs 2 and 4**, *open black arrows*). A characteristic morphologic feature was the seemingly continuous transition (**Fig 4**, *white asterixes*) between the "ribosome complex-like structure" (*open black arrows*) and a multifolded membrane complex of larger dimensions (*trans*-Golgi network?) (*open white arrow*). This transition zone bears visual resemblance to the morphologically continuous transition between a di-gyroid (lattice parameter = 140 nm) (*inset, upper half*) and a mono-gyroid (lattice parameter = 50 nm) (*inset, lower half*) cubic membrane of the endoplasmic reticulum of differentiating sieve elements (Behnke, 1968; Landh, 1996). One may further note the general resemblance between the cubic endoplasmic reticulum with a small lattice parameter (*inset, lower half*) and the new "ribosome complex-like structure" (*open black arrows*).

In this study, a distinct interface between the new "ribosome complex-like structure" and the tonofilament bundles was

conspicuous by its absence. Instead, one may be led to believe that a direct transition between these two entities may take place during the differentiation process (**Fig 4**). We therefore here propose that the new "organelle or branched tubular structure", corresponding to the "lamellar bodies" of classical electron micrographs, takes active part in the formation of the stratum corneum intercellular lipid matrix, whereas the "ribosome complex-like structure" is responsible for the formation of the keratin network, i.e., the keratinization, of the stratum corneum cells.

Figure 5 shows a folded multilamellar continuous structure (*solid white arrow*) adjacent to an apparent active site of skin barrier formation. A similar multilamellar OD pattern could be found in the intercellular space (solid black arrow), indicating a possible continuity between the "continuous multilamellar structure" and the intercellular lipid matrix. If the "membrane folding model" (Norlén, 2001) holds the folded multilamellar continuous structure (**Fig 5**, *solid white arrow*) would be a representation of the terminally differentiated (i.e., multilamellar) lipid matrix of the "tubuloreticular cisternal membrane system" (i.e., the contiguous lamellar bodies and deep invaginations of the cell surface) (Elias *et al*, 1998) at the stratum granulosum/stratum corneum interzone, and thus, in fact, be a representation of the lipid matrix of the intercellular space. In contrast, the presence of this extended "continuous multilamellar structure" "within" the cytoplasmic space of the transition cells is not compatible with the idea of discreet "lamellar bodies".

CONCLUSIONS

Cryotransmission electron microscopy of vitreous epidermal sections may yield new information about the biologic processes involved in skin barrier formation. This is already evident at the macroscopic level, as, by direct comparison with epidermal cryo-electron micrographs, classical electron micrographs express artifactual "empty" areas of significant size and generally exhibit a reduced degree of ultrastructural complexity.

Four characteristic new features of apparent active sites of skin barrier formation have been presented. These are: (i) the often clearly nonlamellar content of structures corresponding to lamellar bodies; (ii) the identification of extended "intracellular" folded multilamellar continuous structures with an OD profile closely corresponding to that of the lipid matrix of the intercellular space; (iii) the "multifolded" appearance of the lipid matrix of the intercellular space; and (iv) a new "ribosome complex-like structure", not preserved in classical electron micrographs.

It has been proposed that during skin barrier formation, a morphologically continuous cubic-like to lamellar membrane transition takes place inside a "branched tubular structure" corresponding to the "lamellar body" system of classical electron micrographs.

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