Gordon Research Conferences

Gordon Research conference on Barrier Functions of Mammalian Skin

Lucca (Barga)
Italy
April 18-23
1999

Chair :
Hans Schaefer
Vice Chair :
Christopher Cullander

Gordon Research Conferences

Conference Program

Barrier Function of Mammalian Skin

April 18-23, 1999
Renaissance Tuscany Il Ciocco Resort
Lucca (Barga), Italy

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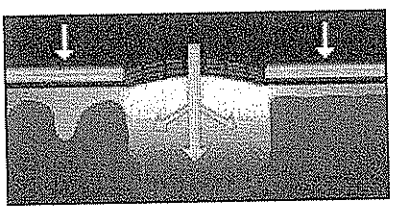

The Gordon Research Conference on the Barrier Function of Mammalian Skin is a biennial event, scheduled for its sixth meeting. The past conferences have been held in the US. This meeting will be organized for the first time in Europe (April 18-23, at the Conference Center Il Ciocco in Barga, Italy). Il Ciocco is now frequently used for Gordon Conferences in Europe. As with all GRC's, registration is limited, and this meeting has always been oversubscribed.

The Barrier Function conference will fully conform to the GRC policy that: the subject matter has been and will be at the frontiers of science; the meeting will be attended by world leaders in the field; emphasis will be on the most recent advances; important and unresolved issues will be identified, defined and analyzed; new research opportunities will be identified; the presentations will allow time for extensive discussion and broad participation; and a diversity of participants will be encouraged, including a broad representation from academia, industry, and government. A significant participation of junior scientists is expected and will be specifically supported.

Sunday 18.4

2:00 - 6:00 pm Registration

6:00 pm Dinner

7:30 - 9:30 pm Evening Session
7:30 - 7:40 Introductory Remarks (**Hans Schaefer**, Clichy/France, Chair) (**Bruno Giannasi**, IL Ciocco, Local Manager)

Optical properties (**Christopher Cullander**, San Francisco/US, Vice Chair)

7:40 - 8:00 **Pierre Corcuff**, Aulnay-sous-Bois/France
"Optical Properties of the Skin Revisited by in vivo Confocal Microscopy"

8:15 - 8:35 **Mark Orazem**, Gainesville/US
"Characterization of Transdermal Delivery *in-vitro* using Optical and Electrochemical Impedance"

8:50 - 9:10 **Robert Imhof**, London/United Kingdom
"Opto-Thermal *in-vivo* Transdermal Diffusion Measurement"

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REGISTRATION OF PARTICIPATION

To Whom it May Concern:

This is to verify that Olga Lopez Serrano who attended the Gordon Research Conference on **Barrier Functions of Mammalian Skin** in Barga, Italy April 18-23, 1999. The payment of $670 was processed for her registation to attend the meeting. Of that amount $335 was towards the registration fee; $235 for the double room lodging; and $150 was towards the meals. This in kind, verifies the amount of **Ms. Lopez Serrano's** registration.

For Dr. Carlyle B. Storm, Director
Date: May 7, 1999

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This is to verify that **Mercedes Cócera** who attended the Gordon Research Conference on **Barrier Functions of Mammalian Skin** in Barga, Italy April 18-23, 1999. The payment of **$670** was processed for her registation to attend the meeting. Of that amount **$335** was towards the registration fee; **$235** for the double room lodging; and **$150** was towards the meals. This in kind, verifies the amount of **Ms. Cócera Núñez 's** registration.

For Dr. Carlyle B. Storm, Director
Date: May 7, 1999

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Barrier Function of Mammalian Skin
Gordon Research Conference
April 18-23, 1999 Lucca (Barga) Italy.

INTERACTION BETWEEN LIPOSOMES AND DELIPIDIZED STRATUM CORNEUM: A STUDY BASED ON HIGH RESOLUTION LOW TEMPERATURE SCANNING ELECTRON MICROSCOPY

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INTRODUCTION

The permeability barrier of skin is mainly located in the lipid lamellar structure of stratum corneum (SC) that mainly consists of ceramides, free fatty acids, cholesterol and cholesterol sulphate [1] It has been demonstrated that mixtures of these lipids are able to form liposomes in the same way that occurred with phospholipids [2].
Although some authors recently claim the advantages of topical application of phospholipid liposomes [3,4], there is not consensus in that the lipid vesicles dermally applied really penetrate into the skin and which would be the cause of such possible penetration. Thus, whereas some authors suggest, that liposomes are not able to penetrate deeper than SC [5] other researchers have reported different effects of these vesicles on the epidermis [6].
In earlier papers we studied the formation of SC lipids liposomes and the interaction of these vesicles with surfactants [7,8]. The main objective of the present work is to study the interaction between SC lipid liposomes and delipidized SC. The SC was delipidized before application of liposomes in order to evaluate the possible improvement of the lipid structure by these vesicles. To this end, double-layer coating for high-resolution low-temperature scanning electron microscopy (HRLTSEM) was used. The main advantage of this method over transmission electron microscopy freeze-fracture is that no cleaning of replicas is needed and, consequently, their possible fragmentation is avoided [9]. The use of this specific technique may be useful to know the ability of the SC lipid liposomes to diffuse into the intercellular spaces of delipidized SC and to be assembled with the lipids of the corneocyte envelope remaining after delipidization.

MATERIALS AND METHODS

Delipidization of SC, Preparation of Liposomes and Incubation with Delipidized SC

The separation of hairless pig epidermis and the isolation of SC were described in previous works [7,10]. In order to delipidize the SC tissue, sheets of native samples were successively extracted for 120 and 60 min with mixtures of chloroform-methanol (2:1, 1:1 and 1:2, v/v). The extracted lipids were concentrated to dryness, weighed, redissolved in chloroform-methanol and then used to prepare liposomes.
In order to prepare SC lipid liposomes the extracted lipid mixture was dried under a stream of N_2 at 25ºC and then hydrated by sonication in TRIS buffer (pH 7.5) at 70 W, 15 min at 70ºC. The final liposomes lipid concentration (10 mg/ml) and its composition were determined using thin layer chromatography coupled to an automated flame detection system (TLC-FID) [7]. The vesicle size distribution of liposomes was determined by dynamic light scattering (DLS) using a photon correlator spectrometer (Malvern Autosizer 4700c PS/MV, Malvern, UK) [8]. The visualization of liposomes was done using a freeze-fracture electron microscopy (FFEM) technique, according to the procedure previously described [11].The replicas were obtained by unidirectional shadowing with 2 nm of Pt/C and 20 nm of C, floated on distilled water and then examined in a Hitachi H-600AB TEM at 75 kV.
In order to study the interaction of liposomes with delipidized SC, this tissue was incubated with liposomes at 25ºC for 18 h and then the tissue was rinsed three times with distilled water and analyzed using HRLTSEM. Given that after delipization by organic solvents the SC was also dehydrated, control samples of SC delipidized were incubated in water alone to evaluate the possible changes induced by rehydration during incubation with the liposome suspension.

High-Resolution Low-Temperature Scanning Electron Microscopy Studies

Three different samples were analyzed: native SC, delipidized SC and SC treated with liposomes after delipidization. Cylindrical pieces of each sample were fixed with 2% glutaraldehyde, cryoprotected with 30% glycerol and mounted on a 3 mm aluminium holder. Thereafter, the SC samples were frozen by plunging into liquid propane and fractured with a microtome knife in a Balzers BAF 300 freeze-etching device at 10^{-7} mbar and a temperature of -110ºC. After etching for 2 min, the fracture plane was coated with 2 nm Pt/C at an angle of 45º, followed by 5-7 nm C at an angle of 90º. Afterwards, the cold samples were immediately cryo-transferred on a Gatan cryo-holder into a Hitachi S-900, in lens field emission scanning electron microscope equipped with a highly sensitive annular YAG-detector for back scattered electrons [9]. Specimens were investigated at -110ºC. The primary accelerating voltage was 10 kV. Images were obtained with the back-scattered electron signal and recorded digitally with a Gatan Digiscan 688 connected to an Apple Quadra 950.
In order to study in detail the pictures obtained by HRLTSEM, the corresponding image analyses was performed. One-dimensional optical density profiles corresponding to the intensity variations vs. distance in the images (nm) were processed.

Freeze-Substitution Experiments

The samples of delipidized SC were cryofixed by high pressure freezing, and were immersed into 5 ml of acetone 2% OsO_4 at -90ºC (8 h), -60ºC (8 h) and -30ºC (8 h). At this temperature the sample was washed in acetone to remove the OsO_4. The samples were then embedded in Epon resin at 4ºC and polymerized 18 h at room temperature and 48 h at 60ºC. Ultrathin sections were stained first with uranyl acetate (5 min) and with lead citrate (5 min) and examined on a Hitachi Model H-600 AB TEM microscope operating at 80 KV.

RESULTS AND DISCUSSION

The lipids extracted from the SC formed liposomes in the composition: 36% ceramides, 27% cholesterol, free fatty acids 24%, cholesteryl esters 4%, triglycerides 3% and cholesteryl sulfate 5%. The dynamic light scattering showed for these vesicles a bimodal distribution curve, in which the size varied in a range between 80 and 150 nm. FFEM was a useful technique to visualize these bilayers, in agreement with previous studies [11]. Figure 1 shows some of these vesicles with diameters of 70, 90 and 150 nm in accordance with the DLS measurements.

Figure 1: Freeze-fracture electron micrograph corresponding to a SC lipids liposome.

Magnifications — 50 nm

The use of HRLTSEM was suitable to visualize SC samples according to the method previously described [9]. The micrograph obtained for native SC is shown in Figure 2

Figure 2: Low-temperature scanning electron micrograph of the native SC. Different structures are indicated as follow: corneocytes as arrow, fracture along the lipid lamellae as arrow heads and fracture across lipid lamellae as open arrows

Magnifications — 300 nm

This Fig. 2 depicts corneocytes (arrows), which are characterized by the particular pattern of keratin entirely filling the interior of these structures and by the absence of cell organelles. Mostly, the fracture plane in the corneocyte lies vertically to the skin surface. In the intercellular space, however, the fracture plane goes along the lamellae of the multilayered lipid organisation resulting in a nice view on the very smooth and relatively flat surface of the SC lipids (arrow heads). Occasionally the bilayers were fractured straightacross, resulting in sharp edges (open arrows), that indicates that lipid matrix was formed by multiple layers.

Two micrographs of delipidized SC at different magnifications are shown in Figures 3-A and 3-B.

Figure 3-A. Low-temperature scanning electron micrograph of delipidized SC, overview.

Magnifications —1400 nm

Figure 3-B: Low-temperature scanning electron micrograph of delipidized SC detailed. The corneocytes are indicated with arrows and the lipid region with arrow heads.

Magnifications — 330 nm

Figures 3-A and 3-B reveal that the granular appearance of the fracture plane corresponding to the corneocytes (keratin tonofilaments, arrows) remained unaltered after the extraction with organic solvents. However, no steps were observed in the lipid domains indicating that only one layer of lipids (possibly that linked with the corneocyte envelopes) persisted between the corneocytes (arrow heads). This indicatives that a great part of the intercellular lipids were extracted by the organic solvents as it was reported in a previous work [12]. The control samples of delipidized SC incubated in water alone were also examined by HRLTSEM (Figure 4) and no differences were found with respect to the SC delipidized without water incubation. Hence, no structural changes induced by rehydration occurred.

Figure 4. Low-temperature scanning electron micrograph of delipidized SC after incubation with water alone.

Magnifications — 1400 nm

Figures 5-A and 5-B show delipidized SC treated with liposomes. Although no changes were found in protein domains, structural changes in the lamellar lipid regions were clearly detected with respect to the delipidized SC. It may be seen that these structures consisted in irregular shapes, smooth steps (arrow) and slightly granular fracture planes. The fact that these structures were not detected in either native or delipidized SC (even after incubation with water alone) indicates that they were due to the treatment with SC lipid liposomes.

Figure 5-A: Low-temperature scanning electron micrograph of delipidized SC after incubation with liposomes. The new structures are indicated with arrows.

Magnifications — 344 nm

Figure 5-B. Higher magnification of an area of micrograph shown in Fig. 5-A where the new structures formed after incubation with liposomes can be observed.

Magnifications — 140 nm

The similarity of the new structures and the corneosomes (modified desmosomes in the SC) found in the delipidized sample and previously described [13] made necessary additional analyses before drawing conclusions on the present findings.In order to clarify this question a structural comparison of the corneosomes and the new structures was performed by means of the image analysis of digitized SEM pictures. To this end, a profile of the new structures (Figure 5-B) was analyzed and compared with that shown in Figure 6 corresponding to desmosomes found in the delipidized SC. Desmosomes exhibited the typical granular appearance due to their intermediate filaments [13].

Figure 6. Low-temperature scanning electron micrograph of a zone of delipidized SC shown desmosomes.

Magnifications — 130 nm

The density profiles for corneosomes and the new structures are shown in Figure 7-A and 7-B, respectively. The right part of each profile corresponds to the background, which was similar in both cases. However, clear differences were detected in the left part of the profiles. Thus, the corneosome profile (Figure 7-A) shows a higher number of peaks than the new structure (Figure 7-B). Furthermore, the peaks for corneosomes showed higher periodicity, being the most frequent distance between peaks 6-10 nm. This distance corresponded to the diameter reported for the filaments in desmosomes by Jeazeiro and Montes [14]. The fact that this distance was not detected in the new structures demonstrated that these structures were not corneosomes.

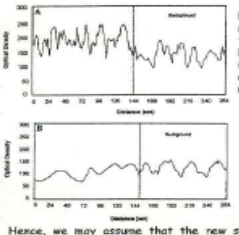

Figure 7. Optical density profiles of representative areas corresponding to the new structures presents in Fig 5-B (Fig 7-A) and to cornesomes in Fig 6 (Fig 7-B). The analyzed areas are indicated in each micrograph as a continuous line.

Hence, we may assume that the new structures were the result of the interaction of the lipids forming liposomes with the lipids of the corneocyte envelope remaining in the intercellular spaces. Although we have not experimental evidence to explain how the new structures were formed, their presence in the intercelular space involves a previous diffusion of the lipids from the liposomes to the intercellular spaces. The diffusion of intact liposomes seems to be an appropriate way to explain the lipid diffusion. However, this fact requires to consider carefully the physico-chemical characteristics of the vesicles (diameter about of 100 nm and phase transition temperature of lipids about 65ºC) and the size of the intercellular spacing of the SC after lipid extraction. Given that freeze-fracture applied to HRLTSEM is not a suitable technique to determine distances (due to the fact that the measurements would be affected by the fracture plane), a freeze-substitution technique for thin sectioning of delipidized SC was used to determine the intercellular spacing. A representative micrograph of delipidized SC is shown in Figure 8. This sample exhibits an organization and structure similar to those described previously for native SC using the same technique [15] . The intercellular space measured in this picture by image analysis was about 80-100 nm. This finding opens the possibility of physical diffusion of liposomes. However, this diffusion does not seem to be possible for native SC, in which case the interlamellar spacing is about 6-13 nm [1] .

Figure 8: TEM micrograph of a thin section of delipidized SC cryofixed and freeze-substituted. The corneocytes are indicated with arrows and the intercellular spaces with arrow heads.

Magnifications — 250 nm

Another possible way of diffusion (in line with the work of Zellmer [16] about the interaction of phosphatidylcholine liposomes with native SC) could be a previous vesicle disaggregation in lipids and diffusion of these lipid molecules through the intercellular channels. Although this could be a good hypothesis to explain the diffusion in native SC, in our case (where a delipidized SC has been used) a diffusion of intact liposomes seems to be more realistic.
In any case the presence of the new structures in our experiments seems to involve a mechanism of re-assembly of the lipid molecules with the lipids linked to the outer face of the envelope. This re-assembly could be associated with the possible restoration of the damaged SC lipid structures by the action of the incubation with SC lipid liposomes. In fact, comparison of pictures of Figure 2 and 5-A shows a certain similarity between the lipid areas of both the native and the treated with liposome samples. In any case this possibility should be confirmed in "in vivo tests" and corroborated in clinical assays.

CONCLUSIONS

The effect of lipid and surfactant vesicles on SC has been studied by a number of authors [6,15-18]. In these works different structural SC changes that disturb the intercellular lipids organization have been reported i.e., formation of water pools and vesicles, deposition of individual molecules, etc. However, no similar structures than those detected in our work have been reported to date. The main reasons could be the following:

- The application of the new tecnique of double layer coating for HRLTSEM was suitable for obtaining images with the advantages of the FFEM but without its drawbacks.
- The use of liposomes formed by lipids directly extracted from SC may facilitate the re-assembly of lipids from liposomes to SC in a similar way that the original arrangement.
- The use of delipidized SC allows not only the observation of the changes produced in the intercellular regions but also a diffusion of lipids as intact liposomes.
- The ability of the SC lipid forming liposomes to be diffused and assembled with the intercellular lipids suggests a potential therapeutic application of these vesicles, in which the container appears to be so important as the contents (encapsulated drugs).

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