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Novel method to observe subtle structural modulation of stratum corneum on applying chemical agents

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ABSTRACT

In the development of functional chemicals such as percutaneous penetration enhancers and cosmetics, the structural evidence at the molecular level in stratum corneum (SC) is highly desirable. We developed a method to observe a minute structural change of intercellular lipid matrix and corneocytes on applying the chemicals to the SC using synchrotron X-ray diffraction technique. The performance of the present method was demonstrated by applying typical chemicals, chloroform/methanol mixture, hydrophilic ethanol and hydrophobic *d*-limonene. From the small- and wide-angle X-ray diffraction we obtained the following results: on applying chloroform/methanol mixture the intercellular lipids were extracted markedly, on applying ethanol the intercellular lipid structure was slightly disrupted, ethanol molecules were taken into the corneocytes and in addition the pools of ethanol seem to be formed in the hydrophilic region of the intercellular lipid matrix in the SC, and on applying *d*-limonene the repeat distance of the long lamellar structure increased by incorporating *d*-limonene molecules, the intercellular lipid structure was slightly disrupted, and the pools of *d*-limonene were formed in the hydrophobic region of the intercellular lipid matrix in the SC.

1. Introduction

The outermost layer of skin, stratum corneum (SC), is composed of corneocytes and intercellular lipid matrix. The structure of the SC plays an important role in the barrier function (White et al., 1988; Bouwstra et al., 1994). To elucidate their molecular mechanisms, sufficient understanding of the structure of the SC is indispensable. The intercellular lipids in the SC form lamellar structures, which are characterized in terms of two orthogonal lattice spacings: one is the lamellar repeat distance and another is the lattice spacing of the lateral hydrocarbon-chain packing structure. The long and the short lamellar structures which have the repeat distances of 13.5 nm and 6 nm, respectively, have been detected by the small-angle X-ray diffraction (SAXD) for the mouse SC (White et al., 1988; Bouwstra et al., 1994; Ohta et al., 2003) and for the human SC (Bouwstra et al., 1991), and the lattice spacings of the hydrocarbon-chain packing structure, 0.41 nm and 0.37 nm, have been observed by the wide-angle X-ray diffraction (WAXD) for the mouse SC (White et al., 1988; Bouwstra et al., 1994) and for the human SC (Bouwstra et al., 1992). Recently it has been proposed that from the study of the temperature change of the simultaneous SAXD and WAXD profiles, the intercellular lipid matrix forms two domains: a domain with hydrophobic character consists of a long lamellar structure with hexagonal hydrocarbon-chain packing in which the lattice spacing is 0.41 nm and a domain with hydrophilic character consists of a short lamellar structure having water layers with orthorhombic hydrocarbon-chain packing in which the lattice spacing is 0.41 nm and 0.37 nm (Hatta et al., 2006). Furthermore, it has been pointed out that very diffuse peaks with the lattice spacing of 1 nm and 0.46 nm observed by the X-ray diffraction are probably caused from the structure of the soft keratin in the corneocytes in the SC (Bouwstra et al., 1994). The SAXD and the WAXD measurements are a powerful tool since the molecular arrangements resulting from the structural modification in the SC at the molecular level can be measured when chemical agents such as penetration enhancers and cosmetics are applied to the SC. It has been pointed out that there exist two potential pathways: one is an intercellular route in which the penetration of chemical agents takes place via the intercellular lipid matrix lying between the corneocytes and the other is a transcellular route in which the penetration takes place across both the corneocytes

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and the intercellular lipid matrix (Suhonen et al., 1999). However, whether the former is the dominant pathway or the both are equivalently important in the penetration is controversial still. Therefore, it is highly desirable to obtain the structural evidence at the molecular level when the chemical agents are applied to the SC.

There are two kinds of the percutaneous penetration enhancers: hydrophilic and hydrophobic ones. For instance, ethanol is one of hydrophilic penetration enhancers (Kurihara-Bergstrom et al., 1990; Kai et al., 1990; Suhonen et al., 1999; Williams and Barry, 2004). It has been pointed out that ethanol may extract some of the lipids from the SC when it is used at high concentration for prolonged times (Kurihara-Bergstrom et al., 1990; Kai et al., 1990). On the other hand a terpene enhancer, *d*-limonene, is well known as a hydrophobic penetration enhancer (Okabe et al., 1988; Cornwell et al., 1994, 1996; Williams and Barry, 2004). So far the effect of these penetration enhancers has been studied by the SAXD and the WAXD. Cornwell et al. (1994) have carried out the WAXD in the SC to study effects of terpene enhancers such as *d*-limonene, nerolidol and 1,8-cineole and found that after 12-h treatment by these terpenes the WAXD intensities for the hexagonal and the orthorhombic hydrocarbon-chain packing structures do not change significantly and on the other hand the broad intensity humps caused by the liquid terpenes incorporated into the SC take place. Cornwell et al. (1996) have performed the SAXD in the human SC to investigate effects of *d*-limonene and 1,8-cineole and found that by treating the SC with these enhancers the intensity for the long lamellar structure decreases and the intensity for the short lamellar structure remains as a shoulder although the WAXD intensities are not modified significantly as mentioned above. As another terpene enhancer, effects of 3% *l*-menthol in 40% ethanol have been studied by the SAXD in the Yucatan micropig SC (Fujii et al., 2003). After treating with *l*-menthol-containing formulation, the SAXD peaks that are probably due to the long lamellar structure decrease. Obata et al. (2006) have carried out the WAXD by treating the hairless rat SC with 2% *l*-menthol in 40% ethanol and found that the intensity from the hexagonal hydrocarbon-chain packing structure decays markedly in comparison with the orthorhombic hydrocarbon-chain packing structure. Furthermore, Bouwstra et al. (1992) have performed the WAXD in the human SC when the lipids are extracted by chloroform/methanol mixture and obtained the result that the intensity for the lattice spacing of 0.37 nm disappeared and that for the lattice spacing of 0.41 nm diminishes markedly. The same behavior has also been pointed out by Cornwell et al. (1994) in the human SC. Besides the terpene enhancers the effects of ethanol on the SC studied by the X-ray diffraction have been little known until now. Under such a situation, it is quite important to establish an X-ray diffraction method which can perform the simultaneous SAXD and WAXD measurements at the same time to know the correlation between the hydrocarbon-chain packing structures and the lamellar structures and furthermore can detect the minute structural changes in both SAXD and WAXD measurements when the chemical agents are applied to SC.

Here we propose a new method for highly sensitive detection of the subtle changes of the structure of the SC by tracking the X-ray diffraction profiles after the application of functional chemical agents. By the use of this method the performance was demonstrated on applying some typical chemical agents to the mouse SC; chloroform/methanol mixture by which the intercellular lipids were extracted markedly, ethanol which is incorporated in the hydrophilic regions in the SC and causes partial disruption of intercellular lipid structure, and *d*-limonene which is incorporated in the hydrophobic regions in the SC and causes disruption of the intercellular lipid structure.

2. Materials and methods

2.1. Sample preparation

Eight-week-old male hairless mice (HR-1, Hoshino, Japan) were used. The mice were treated in accordance with the International Guiding Principles for Biomedical Research Involving Animals published by the Council for the International Organization of Medical Science and the Regulations on Animal Care and Welfare at SPring-8. The skin was separated from a euthanized hairless mouse. After removal of the subcutaneous fat, the skin was soaked with 0.1% trypsin in phosphate-buffered saline solution (pH 7.4) at 4 °C for 16 h. After incubation for another 4 h at 37 °C, the SC was separated from the skin. The SC was then treated with 0.1% trypsin inhibitor, rinsed in distilled water, and dried under vacuum maintained with a rotary pump. Hydration of the SC samples was performed as follows. First, the dried samples were fully hydrated by immersion in water. Second, they were kept in a closed vessel for a few hours at 4 °C. Third, the samples were dehydrated under a stream of dry nitrogen gas until they reached the desired hydration of about 25 wt%, where the water content was defined as 100 (weight of water/[weight of dry SC + weight of water])%. Because of the gradual dehydration process, adjustment of the water content was rather easy in the hairless mouse SC, and the water content was estimated by weighing the sample before and after hydration. Fourth, a piece of the sample (about 3–5 mg) was put into a sample cell for the X-ray diffraction measurement.

2.2. Small- and wide-angle X-ray diffraction

The experiments were performed at BL40B2 (Structural Biology II Beamline) of SPring-8 (Hyogo, Japan). The details of the beamline have been described elsewhere (Miura et al., 2000). The X-ray diffraction profiles were recorded using an imaging plate system (R-AXIS VII; Rigaku, Tokyo, Japan) with a 30 cm × 30 cm area. The X-ray wavelength was 0.1 nm and the sample-to-detector distance was about 400 mm. The scattering vector $S = (2/\lambda)\sin(2\theta/2)$ was calibrated from the lattice spacing ($d = 5.838$ nm; d is the lamellar repeat distance) of a silver behenate crystal at room temperature (Huang et al., 1993), where 2θ is the scattering angle. The measurements were performed every 300 s. The exposure time of the X-ray beam for the SAXD and the WAXD was 30 s. At the SPring-8 a top-up operation was carried out and then the synchrotron radiation light was maintained with constancy of 0.1%. Therefore we could perform the high-resolution observation of the successive changes of the diffraction profiles. The diffraction pattern was circular-averaged to obtain a radial intensity profile. All the experiments were performed at ca. 25 °C. The radiation damage was only small as pointed out previously (Hatta et al., 2006).

2.3. Sample cell and its performance

A sample cell made by us is schematically shown in Fig. 1. Now the cell can be available from SPring-8 Service Co. Ltd. (Hyogo, Japan). A stratum corneum sample was embedded in a central hollow with cylindrical shape surrounded by fine mesh which was used to sustain the sample and also to flow solution without clogging. The fine mesh was composed of glass microfibre filter (grade: GMF 150) purchased from Whatman plc (UK). The diameter of the hollow was about 1.5 mm and the thickness of the hollow was 1.5 mm. The front and the rear surfaces of the cell were sealed by a pair of polyimide thin films with the thickness of 7 μm. A stratum corneum sample was suspended at the middle of the sample cell so that the surroundings of the sample were always filled with sufficient solution. The incident X-ray beam impinged through the front surface. The solution was applied through the mesh. In many