Regular Article, Drug Development and Industrial Pharmacy

Analysis of Hair Follicle Penetration of Lidocaine and Fluorescein isothiocyanate-Dextran 4 kDa using Hair Follicle-Plugging Method

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ABSTRACT

Skin appendages including hair follicles and the stratum corneum are beginning to be recognized as important permeation pathways for the skin permeation of drugs, but their detailed role is not yet clear. To investigate the contribution of hair follicles to drug permeation, we conducted skin permeation tests by controlling the hair follicle contribution with a hair follicle-plugging method. Lidocaine (LC) and fluorescein isothiocyanate-dextran 4 kDa (FD-4) were selected as model drugs and pig ear skin was used as model skin. Skin permeabilities of ionized LC and FD-4 decreased with hair follicle-plugging, whereas no change was observed for the skin permeation of unionized LC. A fairly good correlation was found for ionized LC and FD-4 between skin permeability and the number of hair follicles plugged. Permeation parameters of model drugs for both skin pathways were calculated utilizing Fick's second law of diffusion. Consequently, the stratum corneum pathway could highly contribute to the permeation of unionized LC, since unionized LC shows markedly high partition to the stratum corneum. In contrast, the hair follicle pathway could contribute to the permeation of ionized LC and FD-4, since these had high distributions to the hair follicle pathway in spite of its very small surface area relative to whole skin surface area. Thus, the hair follicle pathway must be important for the skin permeation of ionized compounds and hydrophilic high molecular compounds. Hair follicle-plugging is also a

useful method for assessing the skin permeability of compounds through the hair follicle pathway.

Key words skin permeation; skin appendage; hair follicle; hair follicle-plugging; stratum corneum

INTRODUCTION

External medicines have been used since ancient times to obtain therapeutic efficacy such as disinfection on the skin surface and epidermis. Currently, transdermal therapeutic systems (TTS) or transdermal drug delivery systems (TDDS) containing active ingredients such as nitroglycerin for the treatment of angina[1], tulobuterol for bronchial asthma[2], fentanyl for cancer pain[3], and rivastigmine for Alzheimer's disease[4], have been developed. These drugs have been reported to be highly permeated through the stratum corneum. In general, the permeation rate of therapeutic drugs through a unit area of a skin appendage is much more rapid than that of the stratum corneum because skin appendages such as hair follicles and sweat glands have morphological features that pass through the skin from the epidermis to the dermis. However, the role of a skin appendage pathway on the penetration of drugs in the skin has been largely ignored since skin appendage area is limited to only 0.1% relative to total skin area[5]. Consequently, the stratum corneum becomes the major percutaneous

transport pathway of drugs.

Corneocyte layers consist of ten to several dozen layers in the stratum corneum, in spite of little change depending on the body site, and the intercellular lipid layer is filled between these corneocytes[6]. Thus, the stratum corneum has a tight structure and lipophilic characteristics, such that lipid-soluble and low molecular (especially under 500 Dalton) compounds are more permeable through skin than water-soluble or high molecular compounds[7]. Most active ingredients for external medicines are lipophilic and have a low molecular less than 500 Dalton. Water-soluble and high molecular compounds are not easily permeated through the stratum corneum; therefore, skin appendages are considered to be relatively important for skin permeation of these compounds. Scheuplein theoretically predicted half a century ago that hair follicles could act as a shunt pathway based on the results of rapid skin permeation of ions and polar substances[8]. Hair follicles must be the primary route in the skin appendage pathway for skin penetration of drugs because the size of hair follicles is larger than that of sweat glands. The upper part of infundibular portion of hair follicles is covered with normally structured stratum corneum, which can be considered as a barrier to the skin permeation of drugs. On the other hand, the lower parts of the infundibulum consist of looser corneocytes, which suggests that this structure must be highly permeable. Furthermore, Lieb *et al.* reported that hydrophilic high molecular compounds are probably permeated through skin appendages[9]. However, it is difficult to separately and

quantitatively evaluate the skin permeability of drugs through different specific permeation pathways, since topically-applied drugs are simultaneously permeated through these different pathways. For this reason, only a few studies have evaluated the contribution of hair follicles to skin permeability.

Several methods were reported to evaluate the skin penetration profiles of drugs through hair follicles: the effect of the hair follicle organ on skin permeability was investigated by comparing cultured hair-free skin obtained from scalded skin with intact skin[10]; the effect of the development rate of the hair follicle organ on skin permeability was investigated by comparing newborn rats with 5-day-old rats[11]; the effect of the number of hair follicles on skin permeability was investigated by comparing various skin sites with different hair follicle densities[12]; the effect of the existence of hair follicles on skin permeability was investigated by comparing hairless rat skin with hair with cultured skin without hair[13]. However, skin permeabilities obtained in the previous methods were not really comparative data under the same barrier condition in the stratum corneum and these methods cannot be directly utilized to evaluate the effects of hair follicles on skin permeability. Recently, a new precise hair follicle-plugging treatment was developed to evaluate site-selective skin permeabilities of drugs[14-17]. However, no one utilized these methods to determine permeation parameters through the stratum corneum and hair follicles. Thus, optimal methods have not been established to determine the role of hair follicles on the skin

permeation of drugs.

In the present study, we focused on the hair follicle pathway and evaluated skin permeability through hair follicles. Hair follicles were plugged with a silicone grease-cyanoacrylate adhesives mixture (SC-mixture) to block the hair follicle pathway in order to calculate skin permeation parameters only through the hair follicle route. Lidocaine (LC, pKa; 7.9[18], M.W.; 234 Da), adjusting to an ionized or unionized form with pH conditions, and fluorescein isothiocyanate-dextran (FD-4, M.W.; 4.4 kDa), having hydrophilic and high-molecular weights, were selected as model drugs and the contribution of hair follicles was evaluated in *in vitro* skin permeation experiments. In addition, skin section fluorescence images were observed to determine whether it was possible to prevent hair follicle penetration of drugs by blocking the hair follicle route with the SC-mixture. Pig ear skin was selected as a permeation membrane because skin permeability and histological characteristics such as structure or hair density in porcine skin closely resembles that of human skin[19-22]. In addition, the pig has ineffective sweat glands and does not sweat so much. Therefore, pig skin is assumed to have a small number of sweat glands and is suitable for evaluating drug permeation through hair follicles.

MATERIALS AND METHODS

Materials Lidocaine hydrochloride (LC-HCl, *M.W.*; 289) and FD-4 were obtained from

Sigma-Aldrich Co., Ltd. (St. Louis, MO, U.S.A.). Nile red was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Sodium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). All other reagents and solvents were of reagent grade or HPLC grade and were used without further purification.

Animals Ear skin sets from domestic crossbred pig [LWD] from three strains; Landrace, Large White, and Duroc were obtained from Saitama Experimental Animals Supply Co., Ltd. (Sugito , Saitama, Japan). Frozen pig ear skin sets were stored at -30°C until used for permeation experiments. Pig ear skin was thawed at 37°C on the day of the experiment. Skin was excised and excess fat was trimmed off. All animal studies were done with the recommendations of the Institutional Board for Animal Studies, Josai University (Sakado, Saitama, Japan). The thicknesses of excised pig ear skin used in this experiment were about 1500 μm.

Hair Follicle-Plugging Process To block the penetration of drugs through hair follicles, the SC-mixture consisted of equal parts of silicone grease (Super Lube® Silicone Dielectric Grease; Synco Chemical Corp., Bohemia, NY, U.S.A.) and α-cyanoacrylate adhesives (Aron Alpha Jelly; Konishi Co., Ltd., Osaka, Japan) with small amounts of nile red being applied to hair follicles. Nile red was used to visualize the skin area treated by the SC-mixture. Thus, hair follicles were plugged (Fig. 1) to prevent the penetration of drugs through follicular

pathways. The total number of hair follicles recognized is about 60 in 1.77 cm2 of excised skin area. All the recognized hair follicles or predetermined numbers (10 - 40) of hair follicle in the area were randomly treated with a small amount of the SC-mixture under stereomicroscopic observation. Furthermore, whole permeation area of skin was also treated with the SC-mixture to prevent the drug permeation.

Fig. 1.

***In Vitro* Skin Permeation Experiments** Excised pig ear skin membrane was mounted in vertical-typed diffusion cells (effective diffusion area: 1.77 cm^2). Then, 1.0 mL distilled water was applied to the stratum corneum side and pH 7.4 phosphate-buffered saline (PBS) was applied to the dermal side (receiver cell volume: 6 mL). After 1 h, distilled water was removed and 1.0 mL donor solution containing LC or FD-4 was applied on the stratum corneum side. LC solution was prepared at two different pH values. The pH values were adjusted to pH 5.0 by 1/30 mM phosphate-buffer and to pH 10.0 by 1/30 mM carbonate-buffer, where ionized LC and unionized LC, respectively, predominantly existed. The concentration of LC at pH 5.0 and 10.0 donor solution was adjusted to 100 mM and 10 mM, respectively, and that of FD-4 was 5 mM in PBS. Permeation experiments were performed at 32°C over 8 h (LC) or 12 h (FD-4) through pig ear skin, while the receiver solution was continuously stirred with a star-head-type magnetic stirrer. At predetermined

times, an aliquot (0.5 mL) was withdrawn from the receiver solution and the same volume of fresh PBS was added to keep the volume constant.

Determination of LC The concentration of LC in the sample was determined using a HPLC system (Prominence; Shimadzu, Kyoto, Japan) equipped with a UV detector (SPD-M20A; Shimadzu). The HPLC conditions were modified from Kang et al.[23]. Briefly, 0.2 mL of LC sample was added to 0.2 mL of acetonitrile containing an internal standard (4-hydroxybenzoic acid n-amyl ester; 20 μg/mL) and was vortex-mixed. After centrifugation at 3600×g at 4°C for 5 min, the resulting supernatant (20 μL) was directly injected into the HPLC system. Chromatographic separation was performed using a Unison UK-C18 (3 μm, 75×4.6 mm i.d.; Imtact, Kyoto, Japan) at 40°C. Mobile phases were 10 mM phosphate buffer (pH 6.5)/acetonitrile (1/1, v/v) and the flow rate was 1.0 mL/min. Detection was performed at UV 230 nm.

Determination of FD-4 The concentration of FD-4 in samples was analyzed using a spectrofluorophotometer (RF 5300PC; Shimadzu) at an excitation wavelength of 490 nm and at a fluorescent emission wavelength of 520 nm.

Confocal Laser Scanning Microscope Observation of Yucatan Micropig Skin After skin permeation tests of FD-4, the donor solution was immediately removed from the skin surface and the skin section was prepared with a cryotome (CM3050S; Leica Microsystems Inc., Tokyo, Japan). The cryostat skin section with the stratum corneum side up was

examined using a confocal laser scanning microscope (CLSM, Fluoview FV1000; Olympus Corp., Tokyo, Japan). FD-4 was excited using an Argon laser at an excitation wavelength of 488 nm.

Analysis of permeation parameters The cumulative amount of drugs permeating through skin into the receptor compartment was plotted against time to obtain the skin permeation profile. The cumulative amount of drugs permeated through a unit area of skin was calculated using excluding the SC-mixture treated area that was measured with a digital camera attached to a microscope (DP21; Olympus Corp.). The steady state flux (J) was estimated from the slope of the linear portion of the profile. From J and donor concentration (C_v), the permeability coefficient (P) was calculated by eq.1,

$$P = \frac{J}{C_v} \qquad (1)$$

In addition, in the two-layered diffusion model, the overall permeability coefficient, P_{tot}, can be represented by addition of the reciprocal of the permeability coefficient through the stratum corneum, P_{sc}, and the reciprocal of that through a viable epidermis and dermis, P_{ved}[24]. Furthermore, considering the hair follicle (hf) fraction against whole skin penetration area, A, the skin model is shown as in Fig. 2, and, therefore, P_{tot} can be expressed according to eq. 2,

$$\frac{1}{P_{tot}} = \frac{1}{(1-A)P_{sc} + A \cdot P_{hf}} + \frac{1}{P_{vsd}} \qquad (2)$$

Fig. 2.

RESULTS

Effect of hair follicle-plugging on pig ear skin permeation of ionized or unionized LC The effect of hair follicle-plugging was investigated on pig ear full-thickness skin permeation of LC. We conducted the hair follicle-plugging method where the SC-mixture was applied to hair follicles on the skin surface (Fig. 1). To evaluate the skin permeabilities of each ionized or unionized LC, LC solution was adjusted at pH 5.0 or 10.0, respectively, and then skin permeation tests were performed. Figure 3a and b show the time course of the cumulative amount of ionized or unionized LC that permeated through non-treatment skin, all hair follicles plugged skin, or whole penetration area-blocked skin. The cumulative amount of LC at pH 5.0 was decreased by hair follicle-plugging, although no change was observed in the cumulative amount of LC at pH 10.0 with hair follicle-plugging. Furthermore, in case of whole penetration area-blocked skin, little skin permeation of LC at pH 5.0 and pH 10.0 was detected.

Fig. 3.

Effect of the number of hair follicles plugged on the skin permeability of ionized LC The skin permeability of ionized LC through pig ear full-thickness skin was dramatically decreased by hair follicle-plugging. We then verified whether there were linear relationships between the skin permeability coefficient of LC and number of hair follicles plugged. The number of hair follicles was estimated by a stereomicroscope observation and there were approximately 60 hairs that could be visually recognized on the effective penetration area (1.77cm^2) on pig ear skin. Therefore, the number of hair follicles plugged was set at 0, 10, 20, 30, and 40, and skin permeation tests using ionized LC adjusted at pH 5.0 were performed after hair follicle-plugging. Figure 4a and b show the time course of the cumulative amount of LC that permeated through hair follicle-plugged skin and the relationships between skin permeability coefficients and the number of hair follicles plugged, respectively. Skin permeabilities of ionized LC decreased with an increased number of hair follicles plugged and a fairly good linear relationship was observed ($r = 0.95$).

Fig. 4

Calculation of skin permeation parameters of ionized and unionized LC

Subsequently, skin permeability coefficients (*P*) of ionized and unionized LC were calculated using Fick's law of diffusion. In the case of skin permeation of drugs through intact skin, both routes of the stratum corneum and skin appendages (mainly hair follicles) must be taken into account. In contrast, hair follicle-plugged skin can provide only the stratum corneum route, since the hair follicle route was blocked by the SC-mixture, under the assumption that other trans-appendage routes such as sweat glands could be ignored. In addition, the viable epidermis and dermis are present under the stratum corneum and hair follicles. Under the above assumptions, permeation parameters of LC through whole full-thickness skin (tot) were calculated from the permeation profile of LC through the intact skin. Furthermore, skin permeation parameters of LC through the stratum corneum pathway (sc + ved) and skin appendage (hair follicle) pathway (hf + ved) were calculated from the permeation profiles of LC through all hair follicle-plugged full-thickness skin and estimated skin permeation profiles of LC through hair follicles, respectively. Skin permeation parameters of LC through hair follicles were estimated from permeation profiles obtained by differences between cumulative amounts of drug permeated through full-thickness skin and that through hair follicle-plugged full-thickness skin. Furthermore, considering the permeation parameter of LC on stripped skin, the permeability of LC through the stratum corneum (sc) or hair follicles (hf) could be calculated. Table 1 shows these results. Skin permeation parameters of unionized LC

could not be calculated because the permeation profile through hair follicle-plugged skin was almost the same as that through non-treatment skin. P_{hf} of ionized LC was 22 fold higher than P_{sc}. In contrast, about a 100 fold difference was found between P_{sc} of ionized and unionized LC, although no noticeable difference was found among P_{tot}, P_{sc}, and P_{ved} of unionized LC. In addition, the hair follicle fraction against whole skin penetration area obtained from permeation parameter analysis of ionized LC was 0.20.

Table 1.

Effect of the number of hair follicles plugged on the skin permeation of FD-4

Whether correlative relationships are observed or not between the skin permeability of a hydrophilic high molecular weight compound, FD-4, and the number of hair follicles plugged was investigated. The number of hair follicles plugged was set to 0, 10, 20, 30, and 40 and skin permeation tests of FD-4 were performed. Figure 5a and b show the time course of the cumulative amount of FD-4 that permeated through hair follicle-plugged skin and the relationship between skin permeability coefficients and the number of hair follicles plugged, respectively. Skin permeabilities of FD-4 decreased with an increased number of hair follicles plugged and a fairly good linear relationship was observed between them ($r = 0.95$). Figure 6 shows skin section images observed by CLSM after the skin permeation test of FD-4.

Although non-treatment skin images indicate that FD-4 can penetrate into hair follicles along hair shafts (about 400 μm depth from the skin surface), fluorescence due to FD-4 was observed only on the skin surface in the case of hair follicle-plugged skin.

Figs. 5 and 6.

Calculation of skin permeation parameters of FD-4 Skin permeation parameters of FD-4 were calculated using the same method for LC. Table 2 shows the results. P_{hf} was 20 fold higher than P_{sc} and the hair follicle fraction against whole skin penetration area was 0.17.

Table 2.

DISCUSSION

The p*K*a value of LC was 7.9. Hence, more than 99.9% and more than 99% of LC at pH 5.0 and 10.0, respectively, exist as an ionized form and unionized form, respectively. Therefore, skin permeabilities of ionized and unionized LC were calculated from the permeation data obtained after topical application of pH 5.0 and 10.0 LC solution, respectively. The occlusion method of an infundibulum of hair follicles with the SC-mixture,

which is the hair follicle-plugging method, decreased the skin permeation of ionized LC and had little effect on the skin permeation of unionized LC. Furthermore, the SC-mixture was considered to be effective in blocking the skin penetration of LC, since ionized and unionized LC could not be detected in skin penetration area-blocked skin. Moreover, the present study utilizing hair follicle-plugging suggests that the hair follicle pathway is the primary permeation route for ionized LC. In addition, skin permeabilities of LC decreased with an increased number of hair follicles plugged and a fairly good linear relationship was observed between them. Slight permeation of ionized LC through skin was confirmed under the condition where all recognizable hair follicles were plugged with the SC-mixture (Fig. 3a). These results raise the possibility that LC could be permeated through the non-plugging part of the stratum corneum, sweat glands, and non-treated hair follicles (unrecognizable hair follicles).

In contrast, constant skin permeabilities were observed with or without hair follicle-plugging at pH 10.0, suggesting that the stratum corneum is the primary permeation pathway for unionized LC. Since skin permeability coefficients of drugs generally depend on their partition coefficients and diffusion coefficients, differences in skin permeabilities through the stratum corneum and hair follicles indicated that partition and diffusion parameters were different between both permeation pathways. Calculated skin permeability coefficients suggest that ionized LC had higher distribution to the hair follicle pathway than

the stratum corneum pathway. Permeability coefficients calculated in the present study indicated that ionized LC permeated through skin due to higher distribution to the hair follicle pathway than that of the stratum corneum pathway. The distribution of ionized LC to the hair follicle pathway is higher than that to the stratum corneum pathway and these results may be due to fact that hair follicles are pores filled with water. Moreover, higher LC-diffusivity in the hair follicle pathway than that of the stratum corneum may indicate that the hair follicle pathway consists of a weak (looser) barrier against the skin permeation of drugs. The skin permeability of ionized LC was very low since LC-distribution to skin was dramatically low in the case of the ionized form relative to that of the unionized form. As a result, the hair follicle pathway with high partition and diffusion properties for penetrants must be the primary route for whole skin permeation of ionized LC, although hair follicle area has a small ratio relative to whole skin area. Hair follicles may be an important skin penetration pathway for many ionized compounds.

Subsequently, the effect of hair follicle-plugging was evaluated on the skin permeation of FD-4. Permeability of FD-4 through hairless rat skin has already been reported[13]; most FD-4 permeated through hairless rat skin was detected not through the stratum corneum pathway, but the hair follicle pathway, since FD-4 is a hydrophilic and high molecular weight compound. Skin permeabilities of FD-4 decreased with hair follicle-plugging and highly correlated with the number of hair follicles plugged, which was

similar to results for ionized LC. FD-4 was confirmed to permeate through the hair follicle route by skin section images observed with CLSM. Fluorescence due to FD-4 was observed on the deep part in hair follicles along the hair shafts and only on the skin surface, with non-treatment skin (intact skin) and hair follicle-plugged skin, respectively. Therefore, hair follicle-plugging with the SC-mixture was an effective method to block the skin permeation of drugs through hair follicles and the present experiments demonstrated that the hair follicle pathway was the primary route for the skin permeation of FD-4. The distribution to hair follicles and diffusivity in hair follicles of FD-4 are higher than those to the stratum corneum, and the hair follicle pathway must be the primary route for the skin permeation of FD-4. These results were similar to that of ionized LC. Thus, it was confirmed that hair follicles play an important role in the skin permeation of hydrophilic and high molecular weight compounds.

The present results revealed that skin permeation experiments utilizing hair follicle-plugging can evaluate skin permeation parameters both for stratum corneum and hair follicle pathways. The hair follicle fraction against whole skin penetration area was calculated to be 0.20 and 0.17 in the case of ionized LC and FD-4, respectively, because skin permeation parameters through hair follicles include the effect of the surrounding area around hair follicles and the hair shafts themselves. Thus, these fractions are thought to be over the actually predicted ratio of skin appendage area. This discussion leads to the consideration

that ionized LC and FD-4 can permeate well through a part of the plugging-area (probably a part deep into the hair follicle). The hair follicle pathway must play an importance role on the skin permeation of hydrophilic, ionized, and high molecular weight compounds in the present study, although further detailed research is desired to calculate accurate skin permeation parameters of drugs.

In conclusion, hair follicle infundibulum plugging with a mixture of silicone grease, cyanoacrylate adhesive, and nile red can be considered to be a new tool for evaluating the hair follicle permeability of drugs by blocking the hair follicle pathway. In addition, analysis utilizing the diffusion equation to predict skin permeation parameters made dividing both permeation pathways through the stratum corneum and hair follicle possible. The present study is useful not only for evaluating the skin permeation of hydrophilic, ionized, or high molecular weight drugs, but also to develop TDDS with hair follicle targeting ability.

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