P-Glycoprotein in skin contributes to transdermal absorption of topical corticosteroids

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Abstract

ATP binding cassette transporters, P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), are expressed in skin, but their involvement in transdermal absorption of clinically used drugs remains unknown. Here, we examined their role in transdermal absorption of corticosteroids. Skin and plasma concentrations of dexamethasone after dermal application were reduced in P-gp and BCRP triple-knockout (*Mdr1a/1b/Bcrp*$^{-/-}$) mice. The skin concentration in *Mdr1a/1b/Bcrp*$^{-/-}$ mice was reduced in the dermis, but not in the epidermis, indicating that functional expression of these transporters in skin is compartmentalized. Involvement of these transporters in dermal transport of dexamethasone was also supported by the observation of a higher epidermal concentration in *Mdr1a/1b/Bcrp*$^{-/-}$ than wild-type mice during intravenous infusion. Transdermal absorption after dermal application of prednisolone, but not methylprednisolone or ethinyl estradiol, was also lower in *Mdr1a/1b/Bcrp*$^{-/-}$ than in wild-type mice. Transport studies in epithelial cell lines transfected with P-gp or BCRP showed that dexamethasone and prednisolone are substrates of P-gp, but are minimally transported by BCRP. Thus, our findings suggest that P-gp is involved in transdermal absorption of at least some corticosteroids *in vivo*. P-gp might be available as a target for inhibition in order to deliver topically applied drugs and cosmetics in a manner that minimizes systemic exposure.

Keywords: Skin, Transporter, Transdermal drug delivery, P-glycoprotein, Topical corticosteroid

Abbreviations

P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; SC, stratum corneum; ABC: ATP-binding cassette; MDR1, multidrug resistance 1; MDCK II, Madin-Darby canine kidney II; BL, basal; AP, apical; ER, efflux ratio; P_{app}, apparent permeability coefficient; LC/MS/MS, high-performance liquid chromatography/tandem mass spectrometry; ESI, electrospray ionization; m/z, mass-to-charge ratios.

1. Introduction

Transdermal drug delivery offers several advantages over conventional oral administration or injection, including the avoidance of first-pass metabolism, the minimization of pain and the possibility of controlling drug release (Prausnitz and Langer, 2008; Schoellhammer et al., 2014). However, skin serves as a physical and biological barrier between the body and the environment to prevent unregulated water loss from the body and percutaneous absorption of xenobiotics (Proksch et al., 2008). The physical barrier is mainly localized in the stratum corneum (SC), which is the outermost layer of skin and consists of anucleate corneocytes and intercellular lipids (Madison, 2003). Various techniques have been proposed to increase the skin permeability by disrupting the physical barrier in the SC in order to promote transdermal drug delivery (Prausnitz and Langer, 2008; Schoellhammer et al., 2014). Skin also functions as a biological barrier containing various metabolic enzymes and drug transporters that mediate detoxification and efflux of xenobiotics (Baron et al., 2001; Schiffer et al., 2003; Ahmad and Mukhtar, 2004; Li et al., 2005; Ito et al., 2008; Svensson 2009; Heise et al., 2010; Hewitt et al., 2013). Therefore, this barrier could also have an important influence on transdermal absorption of therapeutic agents, though the mechanisms involved remain to be fully clarified.

Among drug transporters, P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), which are encoded by *MDR1* (or *ABCB1*) and *ABCG2* genes, respectively, in humans, and belong to the ATP binding cassette (ABC) superfamily have been identified in epidermal keratinocytes of both human and mouse (Sleeman et al., 2000; Baron et al., 2001; Triel et al., 2004). Skazik et al. (2011) reported the expression of P-gp in basal epidermis and skin appendages including blood vessels in human. Hashimoto et al. (2013) have also shown that P-gp and BCRP are expressed in the basal layer of epidermis in human, and in dermal endothelial cells of both human and mouse. These two ABC transporters may play a central role in the transdermal absorption of their typical substrate rhodamine 123 (2-[6-amino-3-imino-3H-xanthen-9-yl] benzoic acid methyl ester), at least in rodents, since the concentrations of rhodamine 123 in the plasma and dermis after dermal application were greatly

reduced in *mdr1a/1b* and *bcrp* gene knockout (*Mdr1a/1b/Bcrp*$^{-/-}$) mice, compared with wild-type mice (Hashimoto et al., 2013). Since these transporters have extremely broad substrate specificity, P-gp and/or BCRP might contribute to the transdermal absorption of a variety of therapeutic drugs and cosmetic components.

Various therapeutic agents that are topically applied to the skin or dermally administered for systemic delivery are known to be substrates of P-gp. These include immunosuppressive drugs, antibiotics, antiviral drugs and corticosteroids such as tacrolimus, erythromycin, acyclovir, dexamethasone, prednisolone and betamethasone (Ueda et al., 1992; Saeki et al., 1993; Kim et al., 1999; Yates et al., 2003; Palmberger et al., 2008) for topical application, and opioid analgesics, psychotropic drug, beta-blockers and estrogens such as fentanyl, buprenorphine, methylphenidate, bisoprolol and estradiol (Dagenais et al., 2004; Suzuki et al., 2007; Kim and Benet, 2004; Tahara et al., 2008) for transdermal delivery. P-gp mediates active secretion of its substrate drugs in liver and kidney (Koziolek et al., 2001; Hoffmaster et al., 2004), and is responsible for active efflux of its substrate drugs across brain endothelial cells and small intestinal epithelial cells (Greiner et al., 1999; Sasongko et al., 2005). Although information on the pharmacokinetic roles of BCRP is relatively limited, permeation of some therapeutic agents across the blood-brain barrier is hindered by BCRP-mediated efflux (Agarwal et al., 2011). In the case of skin, however, transdermal permeation of most therapeutic agents applied to the dermal surface is believed to occur mainly by passive diffusion, and the contribution of xenobiotic transporters to overall dermal disposition is mostly unknown and believed to be minor (Mitragotri et al., 2011). In addition, ABC transporters other than P-gp or BCRP are also expressed in the skin (Schiffer et al., 2003; Li et al., 2005; Markova et al., 2009; Heise et al., 2010; Kudo et al., 2016), and exhibit wide range of substrate specificity, so that it is difficult to estimate the contribution ratio of each transporter to overall dermal drug transport. Thus, it is challenging to demonstrate the involvement of these two ABC transporters in transdermal absorption of therapeutic agents *in vivo*.

The purpose of the present study is to clarify the role of P-gp and BCRP in transdermal absorption of therapeutic agents. Since P-gp and BCRP accept therapeutic agents with a variety of molecular sizes as substrates, and passive diffusion of solutes through the skin is hindered by larger molecular size, we focused on corticosteroids as transdermally administered P-gp substrate drugs with relatively large molecular weight in the present study. To examine the involvement of P-gp and/or BCRP in dermal absorption, dexamethasone and other corticosteroids were transdermally administered, and their concentration profiles in plasma and skin were compared in wild-type and *Mdr1a/1b/Bcrp*$^{-/-}$ mice. We also conducted a transcellular transport study in epithelial cell lines stably transfected with genes encoding P-gp and BCRP to confirm transporter-mediated permeation of those drugs.

2. Materials and methods

2.1 Materials

Dexamethasone was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Prednisolone, ethinyl estradiol and elacridar were obtained from Sigma Chemical Co. Ltd. (St. Louis, MO). Itraconazole, Ko143 and methylprednisolone were obtained from LKT Laboratories, Inc. (St. Paul, MN). Prednisone was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals were commercial products of analytical grade.

2.2 Animals

Seven- to nine-week-old male FVB (wild-type) mice and *Mdr1a/1b/Bcrp*$^{-/-}$ mice were purchased from CLEA Japan Inc. (Tokyo, Japan) and Taconic Biosciences, Inc. (Hudson, NY), respectively. The mice were kept in a temperature- and light-controlled environment with standard food and tap water provided ad libitum. Animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals in Kanazawa University. At 72 h before transdermal absorption experiments, mice were anesthetized with a subcutaneous injection of sodium pentobarbital, and the fur over the abdominal skin was removed using an electric hair clipper and depilatory cream. The

chemical components of the cream are thioglycolic acid, cetearyl alcohol, polyoxyethylene lauryl ether, polyoxyethylene cetyl ether, butylene glycol, ethanol, squalene, paraffin, sodium guaiazulene sulfonate, calcium hydroxide and sodium hydroxide. There is no report of their interaction with P-gp or BCRP.

2.3 Transdermal absorption study

Experiments were performed as previously reported with minor modifications (Hashimoto et al., 2013). Briefly, the test drug was dissolved in dimethyl sulfoxide (dexamethasone, prednisolone and methylprednisolone: 50 mg/mL, ethinyl estradiol: 10 mg/mL), and the drug solution (30 μL) was applied to a patch (9 mm diameter, Torii Pharmaceutical Co. Ltd., Tokyo, Japan). Under ether anesthesia, the abdominal skin was stripped with vinyl tape (Nichiban Co. Ltd., Tokyo, Japan), and two patches were applied to abdominal sites. Thus, the dose of dexamethasone, prednisolone and methylprednisolone was 2.4 mg/cm^2, and that of ethinyl estradiol was 0.47 mg/cm^2. At various intervals after dermal application of drug, blood was collected from the tail vein and centrifuged to obtain plasma. After the last blood sampling, mice were sacrificed under ether anesthesia, and the abdominal skin at the location of the patch and brain were collected. In some experiments, the skin was divided into epidermis and dermis as described previously (Surber et al., 1990). Briefly, the skin was wrapped in aluminum foil, put between two glass plates and warmed at 55 ^{o}C for 1 min, and then the epidermis was stripped off with the tip of an injection syringe (18G, Terumo, Tokyo, Japan).

2.4 Intravenous infusion study

Dexamethasone dissolved in saline (50 μg/mL) was injected into the jugular vein at a rate of 100 ng/min. This dose was set to reach the plasma concentration comparable with that observed after the topical administration. At various intervals during intravenous infusion, blood was collected from the tail vein and centrifuged to obtain plasma. At 6 h, several tissues including skin, ear, foot and brain were also excised. The distribution of dexamethasone was represented as the tissue-to-plasma

concentration ratio (μL/mg tissue), which was calculated by dividing the distributed amount of dexamethasone in the tissues (ng/mg tissue) by the plasma concentration at 6 h (ng/mL). Abdominal skin tissues were divided into epidermis and dermis, and the epidermis-to-dermis concentration ratio was also calculated.

2.5 Transport studies in LLC-MDR1 and MDCKII/BCRP/PDZK1 cells

LLC-PK1 cells stably expressing MDR1 (LLC-MDR1 cells) were previously constructed (Schinkel et al., 1995). MDCKII cells stably expressing both BCRP and PDZK1 (MDCKII/BCRP/PDZK1 cells) were also previously constructed (Shimizu et al., 2011). Transcellular transport studies were performed as previously described with minor modifications (Schinkel et al., 1995; Shimizu et al., 2011). Briefly, cells were seeded in Transwell polycarbonate inserts (3 mm pore size, 12 mm diameter; Corning Life Sciences, Edison, NJ) at a density of 3×10^5 cells/well. After 3 days of culture, the cell monolayers were washed with transport buffer (Hanks' balanced salt solution, pH 7.4). Drugs were dissolved in the same buffer (10 μmol/L), with or without inhibitors, and the solution was added to the basal (BL) or apical (AP) chamber. At the designated times, a 100 μL aliquot was collected from the opposite side and replaced with an equal volume of fresh buffer. The efflux ratio (ER) of a drug was calculated as the ratio of the apparent permeability coefficient (P_{app}) in the BL-to-AP direction to that in the AP-to-BL direction, where P_{app} was calculated as the slope of the regression line in the transport time profile of the drug divided by the initial drug concentration in the donor chamber and the cell monolayer surface area (1.1 cm^2).

2.6 Measurement of drug concentration

For the analysis of dexamethasone, prednisolone and methylprednisolone, the plasma and receptor samples were mixed with an equal volume of acetonitrile and 3 volumes (v/v) of the internal standard (500 ng/mL prednisone) dissolved in acetonitrile. The tissue samples were homogenized in 10 volumes (w/v) of acetonitrile containing internal standard using Shake Master NEO (Bio Medical

Science, Tokyo, Japan). After centrifugation, the supernatant was mixed with an equal volume of 10 mmol/L ammonium formate. The supernatant was subjected to high-performance liquid chromatography/tandem mass spectrometry (LC/MS/MS) with a reverse-phase column (Capcell pak MGII C18, 2.1 mm × 50 mm, particle size of 5 μm; Shiseido, Tokyo, Japan). The column was eluted at 0.25 mL/min with a mixture of 10 mmol/L ammonium formate and acetonitrile (2:3, v/v) at 40°C. The LC/MS/MS system was operated in the electrospray ionization (ESI)-positive mode. The mass-to-charge ratios (m/z) were 393.3 and 373.2 for dexamethasone, 361.2 and 147.2 for prednisolone, 375.2 and 357.3 for methylprednisolone, and 359.2 and 341.2 for prednisone. For the analysis of ethinyl estradiol, the samples were prepared as described above using dexamethasone as an internal standard. The supernatant was analyzed in the ESI-negative mode and eluted with a mixture of water and acetonitrile (3:2, v/v). The m/z ratios were 295.1 and 144.8 for ethinyl estradiol, and 391.1 and 361.3 for dexamethasone.

2.7 Determination of mRNA by real-time polymerase chain reaction

Total RNA was extracted from the skin of a mouse (male, 7 weeks), and complementary DNA was synthesized using oligo $(dT)_{12–18}$ primer, deoxynucleotide triphosphate mix, reverse transcriptase buffer, and MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, California). Quantification of mRNAs coding xenobiotic transporters was performed in a mixture of cDNA aliquot, forward and reverse primers, and THUNDERBIRD SYBR qPCR Mix using an Mx3005P (Agilent Technologies, Santa Clara, California). The sequences of forward and reverse primers and the sizes of amplicons are shown in Table S1 in Supplementary Material. The polymerase chain reaction conditions were initiated by template denaturation at 95°C for 15 min, followed by 40 cycles of 10 s of denaturation at 95°C, 30 s of combined primer annealing/extension at 60°C. The expression levels of mRNA were normalized to an endogenous control, acidic ribosomal phosphoprotein P0 (36B4).

2.8 Statistical analysis

The statistical significance of differences was determined by means of Student's *t*-test with $p < 0.05$ as the criterion of significance.

3. Results

3.1. Transdermal absorption of dexamethasone

P-gp and BCRP are functionally expressed in skin (Hashimoto et al., 2013), but so far there is no evidence that these ABC transporters are involved in the transdermal absorption of topical drugs used to treat dermatological diseases. In the present study, we first examined the effect of gene deletion on the transdermal absorption of the topical steroidal anti-inflammatory drug dexamethasone (Fig. 1). The plasma concentration of dexamethasone after dermal administration in *Mdr1a/1b/Bcrp*$^{-/-}$ mice was significantly lower than that in wild-type mice at all time points examined (Fig. 1A). At 6 h after dermal administration, the concentration of dexamethasone in the skin at the location of the patch in *Mdr1a/1b/Bcrp*$^{-/-}$ mice was also significantly lower than that in wild-type mice (Fig. 1B). Since skin has a multilayer structure, we determined the concentrations of dexamethasone in epidermis and dermis separately at 6 h after transdermal administration. The concentration of dexamethasone in epidermis was similar in wild-type and *Mdr1a/1b/Bcrp*$^{-/-}$ mice, whereas that in dermis of *Mdr1a/1b/Bcrp*$^{-/-}$ mice was lower than that in wild-type mice (Fig. 1C). However, quantitative discussion should be carefully performed because the difference in dexamethasone concentration in dermis between wild-type and *Mdr1a/1b/Bcrp*$^{-/-}$ mice (Fig. 1C) was not so remarkable, compared with that in whole skin (Fig. 1B). This was at least partially due to the loss of dexamethasone during the heat separation, because the recovery of dexamethasone during the separation was estimated to be 74 and 81% in wild-type and *Mdr1a/1b/Bcrp*$^{-/-}$ mice, respectively. As a control study, the distribution of dexamethasone was also examined in the brain, where these transporters act as a barrier against entry of their substrates from the systemic circulation. The brain concentration of dexamethasone in *Mdr1a/1b/Bcrp*$^{-/-}$ mice was much higher than that in wild-type mice (Fig. 1D), confirming that P-gp and/or BCRP-mediated efflux of dexamethasone from the brain occurs *in vivo*. This is consistent with

previous reports showing that P-gp limits the entry of dexamethasone into the brain (Schinkel et al., 1995; Uchida et al., 2011), although the involvement of BCRP has not been reported.

3.2. Gene deletion of P-gp and BCRP minimally affects systemic elimination, but increases distribution of dexamethasone to epidermis

Although the difference in plasma concentration profile of dexamethasone after dermal application between wild-type and *Mdr1a/1b/Bcrp*$^{-/-}$ mice (Fig. 1A) can be explained by a difference in transdermal absorption, it is also possible that there might be a difference in systemic elimination. To examine this possibility, the plasma concentration profile of dexamethasone was measured during intravenous infusion of dexamethasone. The plasma concentration profile of dexamethasone during intravenous infusion was almost the same in the two strains (Fig. 2A), indicating that any difference in systemic elimination is minimal. The tissue concentrations were also measured at 6 h after the start of infusion. The tissue-to-plasma concentration ratio in the brain of *Mdr1a/1b/Bcrp*$^{-/-}$ mice was much higher than that in wild-type mice (Fig. 2D), whereas that in side abdominal skin showed no significant difference between wild-type and *Mdr1a/1b/Bcrp*$^{-/-}$ mice (Fig. 2B). The tissue-to-plasma concentration ratio in the skin samples from other sites (abdomen, back, glabella and neck) as well as ear, forefoot and hind foot was also similar between the two strains (data not shown). On the other hand, when we separately evaluated dexamethasone concentrations in the epidermis and dermis of side abdominal skin, a significantly higher concentration was observed in epidermis of *Mdr1a/1b/Bcrp*$^{-/-}$ mice, compared with that in wild-type mice (Fig. 2C). The epidermis-to-dermis concentration ratio of dexamethasone in *Mdr1a/1b/Bcrp*$^{-/-}$ mice (1.52 ± 0.07) was also higher than that in wild-type mice (0.948 ± 0.098).

3.3. Transport of dexamethasone by P-gp, but not BCRP

To determine whether dexamethasone is a substrate of P-gp and/or BCRP, we examined the transcellular transport of dexamethasone across LLC-MDR1 and MDCKII/PDZK1/BCRP cell monolayers. The permeation of dexamethasone in the BL-to-AP direction across LLC-MDR1 cells was higher than that in the AP-to-BL direction (Fig. 3A). This transcellular transport in the AP-to-BL direction was increased in the presence of the P-gp inhibitor itraconazole (Fig. 3A). The ER value in the presence of itraconazole was lower than that in its absence, suggesting that dexamethasone is a substrate of P-gp. The permeation of dexamethasone in the BL-to-AP direction across MDCKII/PDZK1/BCRP cells was similar to that in the AP-to-BL direction, and the BCRP inhibitor Ko143 had no effect on the transcellular transport (Fig. 3B).

3.4. Transdermal absorption and P-gp-mediated transport of other topical steroids

To examine the involvement of P-gp in the transdermal absorption of other topical drugs, we further examined the transdermal absorption of prednisolone, methylprednisolone and ethinyl estradiol in wild-type and *Mdr1a/1b/Bcrp*$^{-/-}$ mice (Fig. 4). The plasma concentration of prednisolone in *Mdr1a/1b/Bcrp*$^{-/-}$ mice was lower than that in wild-type mice, showing significant differences at 0.5, 4 and 6 h after dermal administration (Fig. 4A). The skin concentration of prednisolone in *Mdr1a/1b/Bcrp*$^{-/-}$ mice was also lower, but the brain concentration in *Mdr1a/1b/Bcrp*$^{-/-}$ mice was higher than that in wild-type mice (Fig. 4B, 4C), as in the case of dexamethasone (Fig. 1B, 1D). This is consistent with the previous report that P-gp decreases the brain distribution of prednisolone (Karssen et al., 2002). On the other hand, the plasma and skin concentrations of methylprednisolone in *Mdr1a/1b/Bcrp*$^{-/-}$ mice were not so much different from those in wild-type mice after transdermal administration (Fig. 4D and 4E), whereas the brain concentration in *Mdr1a/1b/Bcrp*$^{-/-}$ mice was much higher than that in wild-type mice (Fig. 4F). The plasma, skin and brain concentrations of ethinyl estradiol after transdermal administration were similar in wild-type and *Mdr1a/1b/Bcrp*$^{-/-}$ mice (Fig. 4G-I).

To directly confirm transport by the ABC transporters, we examined the transcellular transport of these three steroids across LLC-MDR1 and MDCKII/PDZK1/BCRP cells monolayers. The transcellular transport of prednisolone and methylprednisolone in the BL-to-AP direction across LLC-MDR1 cells was higher than that in the AP-to-BL direction (Fig. 3C and 3E). Effect of more potent P-gp inhibitor elacridar on the directional transport was also examined to clearly evaluate whether P-gp transports these steroid or not. In the presence of elacridar, AP-to-BL transport of these drugs was increased, whereas BL-to-AP transport was decreased (Fig. 3C and 3E). The ER values of prednisolone and methylprednisolone in the presence of elacridar were lower than those in its absence. On the other hand, ethyinyl estradiol exhibited no directional transport across LLC-MDR1 cells (Fig. 3G). Transport of prednisolone and methylprednisolone in the BL-to-AP direction across MDCKII/PDZK1/BCRP cells was slightly higher than that in the AP-to-BL direction, but Ko143 did not influence the transcellular transport (Fig. 3D and 3F). Ethyinyl estradiol did not exhibit directional transport across MDCKII/PDZK1/BCRP cells, and Ko143 had no effect (Fig. 3H).

3.5. Gene deletion of P-gp and BCRP minimally affects gene expression of other ABC xenobiotic transporters in skin

To examine possible secondary effects of gene deletion on the expression of other ABC xenobiotic transporters in *Mdr1a/1b/Bcrp*$^{-/-}$ mice, the mRNA levels for multidrug resistance associated protein (MRP) 1 to MRP5 were measured in the skin of wild-type and *Mdr1a/1b/Bcrp*$^{-/-}$ mice. Gene expression of MRP1, 3, 4 and 5 was similar in wild-type and *Mdr1a/1b/Bcrp*$^{-/-}$ mice, while expression of MRP2 was not detected in either of the strains (Fig. 5). Thus, gene expression of these MRPs seems not to be affected by knockout of *mdr1a/1b/bcrp* genes.

4. Discussion

Transdermal absorption of therapeutic agents, including topical corticosteroids, has been considered to be mediated principally by passive diffusion (Mitragotri et al., 2011). On the other hand, xenobiotic

transporters including P-gp and BCRP have been identified in both mouse and human skin (Sleeman et al., 2000; Baron et al., 2001; Triel et al, 2004; Skazik et al., 2011; Hashimoto et al., 2013), although their roles in the skin are largely unknown. Our present results provide the first evidence that P-gp contributes to transdermal absorption of topical corticosteroids, including dexamethasone. (i) First, the plasma and skin concentrations after topical application of dexamethasone in *Mdr1a/1b/Bcrp*$^{-/-}$ mice were lower than those in wild-type mice (Fig. 1A, 1B). (ii) The plasma concentration profile of dexamethasone after intravenous administration was similar in the two strains (Fig. 2A), indicating that the difference in plasma concentration profile after dermal application (Fig. 1A) cannot be explained by a difference in systemic elimination, and therefore could be due to a difference in transdermal absorption. (iii) Directional transport of dexamethasone in the BL-to-AP direction was observed in LLC-MDR1cells (Fig. 3A), but not in MDCKII/PDZK1/BCRP cells (Fig. 3B), and this transport was reduced in the presence of the P-gp inhibitor itraconazole (Fig. 3A). (iv) When skin was separated into epidermis and dermis, the concentration of dexamethasone was reduced only in dermis in *Mdr1a/1b/Bcrp*$^{-/-}$ mice (Fig. 1C), and this is consistent with the finding reported for the typical P-gp substrate rhodamine 123 (Hashimoto et al., 2013). (v) Plasma and skin concentrations after topical application of another P-gp substrate corticosteroid, prednisolone, were also lower in *Mdr1a/1b/Bcrp*$^{-/-}$ mice than in wild-type mice (Fig. 4A, 4B), suggesting that P-gp-mediated transdermal absorption of topical drugs may not be specific to dexamethasone. However, the reduction in the transdermal absorption in *Mdr1a/1b/Bcrp*$^{-/-}$ mice was only partial in the cases of dexamethasone and prednisolone (Fig. 1A, 4A), suggesting possible involvement of other mechanism(s) than P-gp in their absorption. In the transdermal administration of steroids, SC was removed by tape stripping because the loss of SC is often observed in the skin of atopic dermatitis. In addition, DMSO was used as a vehicle for steroids topically applied using the patch to control the dose and applied area as previously reported (Ito et al., 2008). As DMSO is known to extract lipid in skin, possible morphological change in the skin cannot be excluded during the transdermal administration. Nevertheless, the present findings provide the first evidence that P-gp expressed in the skin plays a

role in transdermal absorption of topically applied corticosteroids.

Among topical therapeutic drugs, corticosteroids are widely used for various skin diseases, and especially are used as first-line drugs for atopic dermatitis (Ference and Last, 2009; Ring et al., 2012). However, the long-term use of topical corticosteroid may cause severe systemic adverse effects, including adrenal suppression, hyperglycemia and glaucoma, as well as local adverse effects including skin atrophy and acne (Hengge et al., 2006). These adverse effects of topical corticoids limit their practical use and may influence patient compliance with the treatment. Therefore, various techniques and formulations have been proposed (Korting et al., 2002; Sul et al., 2013; Siddique et al., 2015) to reduce absorption of topical corticosteroids into the systemic circulation and/or improve their retention in the skin. Our present results suggest that inhibition of P-gp may decrease the distribution of topical corticosteroid to the systemic circulation after dermal application, and thus may improve retention in skin. This hypothesis is supported by the present findings that the concentrations of dexamethasone in plasma and dermis after dermal application were decreased in *Mdr1a/1b/Bcrp*$^{-/-}$ mice, but the concentration in the epidermis was similar in wild-type and *Mdr1a/1b/Bcrp*$^{-/-}$ mice (Fig. 1). Thus, P-gp inhibitors may be useful to minimize the systemic side effects of topical corticosteroids without reducing their efficacy in skin, provided that the inflammatory target is localized within the epidermis. P-gp inhibitors are present in topical therapeutic agents for skin diseases, such as ketoconazole, erythromycin and tacrolimus, and excipients for topical formulations, such as polyethylene glycol-300 and pluronic P85 (Batrakova et al., 2001; Ekins et al., 2002; Hugger et al., 2002; Kishimoto et al., 2014). Therefore, the modulation of P-gp activity by the coadministration of P-gp inhibitor may be a feasible technique to control the transdermal absorption of topical corticosteroids. Further work will be needed to test this idea.

In the present study, the concentrations of methylprednisolone and ethinyl estradiol in both skin and plasma of *Mdr1a/1b/Bcrp*$^{-/-}$ mice were similar to those in wild-type mice *in vivo* (Figs. 1 and 3), although methylprednisolone was found to be a P-gp substrate in LLC-MDR1 cells *in vitro* (Fig. 3E). Thus, P-gp substrates identified in *in vitro* transport studies may not necessarily be efficient substrates

of P-gp in the skin *in vivo*. Indeed, in preliminary studies, we also examined transdermal absorption of other P-gp substrate drugs, including cyclosporin A, itraconazole and ketoconazole, but observed only minimal differences between wild-type and *Mdr1a/1b/Bcrp*$^{-/-}$ mice (data not shown). The lack of influence of P-gp gene knockout on transdermal absorption of methylprednisolone could be explained by high skin permeability of methylprednisolone compared to dexamethasone and prednisolone, resulting in a lower contribution of P-gp to overall transdermal transport. Indeed, the concentration of methylprednisolone in skin after dermal application (~800 ng/mg tissue) was much higher than that of dexamethasone and prednisolone (100~200 ng/mg tissue), even though the same dose of each drug was topically applied (Figs. 1B, 4B and 4E). Thus, P-gp may significantly influence the transdermal absorption of only topical therapeutic drugs whose permeability is relatively low. On the other hand, P-gp may also contribute to the skin distribution of other therapeutic drugs after systemic administration. For example, the SC-to-serum concentration ratio of fluconazole after oral administration in humans was reported to be higher than that of ketoconazole (Harris et al., 1983; Faergemann and Laufen, 1993). The brain distribution of ketoconazole in P-gp knockout mice after intraperitoneal injection was higher than that in wild-type mice, but that of fluconazole after oral administration were similar in the two strains (Yasuda et al., 2002; von Moltke et al., 2004), suggesting that ketoconazole, but not fluconazole is a substrate of P-gp *in vivo*. Since P-gp regulates transport from epidermis to dermis, and that from dermis to circulation, the higher SC-to-serum concentration ratio of fluconazole may be explained in terms of lower transport efficiency by P-gp, leading to lower transport from epidermis to the circulation.

In *Mdr1a/1b/Bcrp*$^{-/-}$ mice, the concentration of dexamethasone in dermis was reduced after topical application (Fig. 1C), whereas that in epidermis was increased after intravenous administration (Fig. 2C), compared with wild-type mice. This observation can be quantitatively explained by a two-compartment model which was previously proposed (Hashimoto et al., 2013) and separated skin into viable epidermis and dermis (Fig. S1, see Supplementary Material for details).

In the present study, we could not evaluate the contribution of BCRP to the transdermal absorption

of topical corticosteroids because the steroids examined in the present study were found not to be BCRP substrates (Fig. 3). However, we have previously reported that the concentrations of rhodamine 123 in skin and plasma of *Mdr1a/1b/Bcrp*$^{-/-}$ mice tended to be lower than those in P-gp knockout (*Mdr1a/1b*$^{-/-}$) mice (Hashimoto et al., 2013), suggesting that BCRP may also be involved in transdermal absorption. Other xenobiotic transporters such as MRPs, carnitine/organic cation transporter 1 (OCTN1) and oligopeptide transporter 2 (PEPT2) were also reported to be functionally expressed in epidermis (Li et al., 2005; Markova et al., 2009; Heise et al., 2010; Kudo et al., 2016). Li et al. (2005) have reported that MRP1 contributes to the efflux of two substrates, fluo3 and grepafloxacin, from skin. Heise et al. (2010) also reported that MRPs are involved in efflux of steroid hormones and contact allergens such as eugenol in human keratinocytes. Markova et al. (2009) found that OCTN1 contributes to the uptake of its substrate antioxidant L-ergothioneine in keratinocytes. These reports, however, did not examine transdermal absorption after topical application of transporter substrates. On the other hand, Kudo et al. (2016) recently reported that PEPT2 contributes to the skin penetration of dipeptide glycylsarcosine in the absorptive direction. Thus, various transporters may be involved in the transport of their substrates in both influx and efflux directions in the skin. Furthermore, since these transporters are expressed in epidermis (Markova et al., 2009; Heise et al., 2010; Kudo et al., 2016), drug transporters may cooperatively play a role in controlling the dermal disposition of drugs and cosmetics, implying that these transporters may be available as targets to control exposure to topically applied compounds in various drug delivery systems.

5. Conclusions

Our present findings indicate for the first time that P-gp contributes to the transdermal absorption of topical corticosteroids, including dexamethasone. Modulation of P-gp activity may be a useful approach to control the retention of P-gp substrates in the dermal epidermis and to decrease their systemic absorption. The present findings thus open up a new strategy for transdermal drug delivery, targeting dermal transporters.

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Legends for figures

Fig. 1. Transdermal absorption of dexamethasone in mice after topical application to tape-stripped skin.

Dexamethasone dissolved in DMSO (30 μL of 50 mg/mL) was applied to two patches for topical administration, and the patches were applied to tape-stripped abdominal skin. The concentration of dexamethasone in plasma (A) was measured in wild-type (open circles) and *Mdr1a/1b/Bcrp*$^{-/-}$ (closed circles) mice. Concentrations in skin (B), epidermis (C), dermis (C) and brain (D) were also measured at 6 h after the start of topical application in wild-type (open columns) and *Mdr1a/1b/Bcrp*$^{-/-}$ (closed columns) mice. Data are expressed as the mean ± S.E.M. (n = 9 and 4 in plasma and tissue samples, respectively). *Significantly different from wild-type mice ($p < 0.05$).

Fig. 2. Plasma concentration and tissue distribution of dexamethasone in wild-type and *Mdr1a/1b/Bcrp*$^{-/-}$ mice during intravenous infusion.

Dexamethasone was intravenously infused at a rate of 100 ng/min. The concentration of dexamethasone in plasma (A) was measured in wild-type (open circles) and *Mdr1a/1b/Bcrp*$^{-/-}$ (closed circles) mice. Tissue-to-plasma concentration ratios in side abdominal skin and brain (B and D, respectively), and concentration in epidermis and dermis of the side abdominal skin (C) were also measured at 6 h after the start of infusion in wild-type (open columns) and *Mdr1a/1b/Bcrp*$^{-/-}$ (closed columns) mice. Tissue-to-plasma concentration ratios were calculated by dividing the distributed amount in the tissue by the plasma concentration at 6 h (B and D). Data are expressed as the mean ± S.E.M (n=4-5). *Significantly different from the wild-type mice ($p < 0.05$).

Fig. 3. Transcellular transport of dexamethasone (A, B), prednisolone (C, D), methylprednisolone (E, F) and ethinyl estradiol (G, H) by P-gp and BCRP.

Transport of dexamethasone (10 μmol/L) in the AP-to-BL (squares) and BL-to-AP (circles) directions was measured across LLC-MDR1 cells (A, C, E, G) and MDCKII/PDZK1/BCRP cells (B, D, F, H).

Transport was examined in the presence (closed symbols) or absence (open symbols) of the MDR1 inhibitor itraconazole (25 μmol/L, panel A) or elacridar (5 μmol/L, panels C, E, G) in LLC-MDR1 cells and the BCRP inhibitor Ko143 (1 μmol/L) in MDCKII/PDZK1/BCRP cells. Each value represents the mean ± S.E.M. (n = 3).

Fig. 4. Transdermal absorption of prednisolone, methylprednisolone and ethinyl estradiol in mice after topical application to tape-stripped skin.

Prednisolone (50 mg/mL), methylprednisolone (50 mg/mL) and ethinyl estradiol (10 mg/mL) dissolved in DMSO (30 μL) were each applied to patches for topical administration, and two patches were applied to tape-stripped abdominal skin. Plasma concentrations of prednisolone (A), methylprednisolone (D) and ethinyl estradiol (G) were measured in wild-type (open circles) and *Mdr1a/1b/Bcrp*$^{-/-}$ (closed circles) mice. Concentrations of prednisolone (B, C), methylprednisolone (E, F) and ethinyl estradiol (H, I) in skin (B, E, H) and brain (C, F, I) were also measured at 6 h after the topical application in wild-type (open columns) and *Mdr1a/1b/Bcrp*$^{-/-}$ (closed columns) mice. Data are expressed as the mean ± S.E.M. (n = 3-4). *Significantly different from wild-type mice ($p < 0.05$).

Fig. 5. Gene expression of various ABC transporters in skin of wild-type and *Mdr1a/1b/Bcrp*$^{-/-}$ mice. The mRNA level for each transporter in wild-type (open columns) and *Mdr1a/1b/Bcrp*$^{-/-}$ (closed columns) mice was quantified by RT-PCR and normalized by that of 36B4. Each point represents the mean ± S.E.M. (n = 3). N.D., not detectable.