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Expression of human solute carrier family transporters in skin: possible contributor to drug-induced skin disorders

SUBJECT AREAS:

DRUG SAFETY
DIAGNOSTIC MARKERS

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Solute carrier (SLC) transporters play important roles in absorption and disposition of drugs in cells; however, the expression pattern of human SLC transporters in the skin has not been determined. In the present study, the expression patterns of 28 human SLC transporters were determined in the human skin. Most of the SLC transporter family members were either highly or moderately expressed in the liver, while their expression was limited in the skin and small intestine. Treatment of human keratinocytes with a reactive metabolite of ibuprofen significantly reduced cell viability. Expression array analysis revealed that S100 calcium binding protein A7A (S100A7A) was induced nearly 50-fold in dermal cells treated with ibuprofen acyl-glucuronide. Determination of the expression of drug-metabolizing enzymes as well as drug transporters prior to the administration of drugs would make it possible to avoid the development of idiosyncratic skin diseases in individuals.

Membrane transport proteins are involved in the transport of endogenous and exogenous compounds across plasma membranes. Transport proteins are largely composed of two families; solute carrier (SLC) transporters and ATP binding cassette (ABC) transporters. While ABC transporters utilize the energy of ATP hydrolysis to transport their substrates across membranes¹, SLC transporters utilize ion or electrochemical gradients such as sodium or proton gradients to transport substrates. Currently 49 ABC transporter subtypes, including a pseudogene, have been identified in humans, and they are divided into seven subfamilies, ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, and ABCG¹. In contrast, more than 384 unique protein sequences, which are divided into 52 distinct families (SLC1 to SLC52), have been identified². Among those transporters, certain subtypes are involved in the transport of drugs. They play an important role in absorption, distribution, and excretion of drugs – essential steps of drug kinetics/pharmacokinetics in human bodies.

SCL21 and SLC22 genes encode organic anion transporting polypeptide (OATP), organic anion transporter (OAT), and organic cation transporter (OCT) family transporters, which mediate absorption, distribution, and excretion of a wide variety of environmental toxins and clinically used drugs, including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatory drugs^{3,4}. While these transporters are expressed in various tissues, their expression is especially important in the small intestine, liver, kidneys, and blood-brain barrier, because drug transporters play an essential role in the absorption, distribution, and excretion in these tissues. Therefore, expression patterns of these and the other transporters in the small intestine, liver, kidneys, and blood-brain barrier have been extensively examined to date. It has been demonstrated that the expression levels of PRPT1, OCTN2, MCT1, and OATP2B1 are relatively higher in the human small intestine⁵. In the human liver, NTCP, OCT1 and OATP1B1 are highly expressed to facilitate uptake of drugs into hepatocytes⁶ and drugs are thereby metabolized by drug-metabolizing enzymes, such as cytochrome P450 and UDP-glucuronosyltransferases expressed in the endoplasmic reticulum of hepatocytes. In human kidneys, various SLC transporters have been reported to be expressed, such as PEPT1, OCT2 and 3, OCTN1 and 2, OAT1 to 4, and URAT1, and to be involved in the excretion and reabsorption of drugs⁷.

Although drugs exhibit therapeutic effects in the body, they can also cause adverse reactions. While drug-induced toxicities can occur in various tissues, drug-induced toxicity in the skin is becoming a matter of great concern due to its severity. Stevens–Johnson syndrome⁸, psoriasis⁹, allergy¹⁰, hypersensitivity syndrome¹¹, Lyell syndrome¹², and photosensitivity¹³ are examples of severe drug-induced toxicity in skin. Increased concentrations of drugs are highly associated with the onset of these adverse reactions to drugs. Therefore, accumulation of drugs and metabolites in the dermal cells can be a determinant of the onset of drug-induced toxicity in skin. While drug transporters expressed in skin cells would play an important role in determining the concentration of drugs and



Table 1 | Primer sequences for human SLC transporters

Subtypes	Forward primers (5' to 3')	Reverse primers (5' to 3')
SLC10A1	CTGAGCGTCATCCTGGTGT	TTCATGTCCCCCTTCATGGC
SLC10A2	TGGCCTTGGTGATGTTCTCC	TGTCGCCATCGACCCAATAG
CDH17	TGGATATGGCCAAGAGGGGA	ATAGGGACTGGACCCTCCAC
SLC15A1	CAAGTGCATCGGTTTTGCCA	CCCGGACATAGTTGTTGCC
SLC15A2	TGATCAAGTCTTGGGTGCC	CCCTGCATTGATGGACAGGT
SLC16A1	CACCAGCGAAGTGCATGGA	TCAAGTTGAAGGCAAGCCCA
SLC16A4	GGGACTTCTACCCGGTTTGG	CATGTGCCTCTGGACCATGT
SLC22A1	TCTGTGCTCTATCAGGGGCT	TGATTCCCATTGCGCCAACA
SLC22A2	GTTGGGCGGAGATATCGGAG	AAGGGAGGCGGGTAGAGATT
SLC22A3	TCCCAACTGGCAAGGAATC	ATACACCACTGCGCTTGTGA
SLC22A4	GTTGAGCCAGGACGTCTACC	AGCCATGGTTGCGAAGAGAA
SLC22A5	TGGCACTCTGGTGGTTTCATC	CTGGGACTTCAACCATCGCT
SLC22A6	TTGGGCTACCTTGCAGACAG	GAATGGGCATCCACTCCACA
SLC22A7	TGCAACTGAGTGGGATCTGG	AGCACCAGGGTACTCACGTA
SLC22A8	ACTCGGGTACTGCTACACCT	CAGGTCACCTGCGGTGTACT
SLC22A9	AGGTTCTGCTCAGATGGTG	CCATGGCCTGGAATCTGTGT
SLC22A11	TTATCTGGGGCCTCCTCTCC	GTGGTCCACTCCACCATCAG
SLC28A3	GGGGAATCGGGCTACAGTTT	CAGCAGTGGAGACTCCGTTT
SLCO1A2	ACCAACGCAGGATCCATCAG	AAAGCCAAACCACCATGCAC
SLCO1B1	ACTGTCTTGCATGTGCTGG	GGCAATTCCAACGGTGTCA
SLCO1B3	GCAGCCCTGTCATTCAGCTA	CAGTTCCTATAAGGAGACAACA
SLCO1C1	ACTCCCATTGAGCCTTGGG	CAGAAAAGGCACAGCTGCAAG
SLCO2B1	TGGGCACAGAAAACACACCT	CGGCTGCCAAAATAGCTCAC
SLCO3A1	CTCCTACATCGACGACCACG	TCTTTTCGGAGAGCATGGC
SLCO4A1	GCGTCACCTACTGGATGAG	TAACCAAGGATGGGAACGGC
SLCO4C1	TTCTGTGCCACACACAAGT	CCAATCCACCAAGCTCCAA
PCFT	GCTGATAGCCATGACTCTCT	CAGAACCATAGCCGATTAGT
RFC	GAGCAGCATCTGGCTGTGCT	GCACGTCCGAGACAATGAAAGT

metabolites in the cells, little is known about the expression pattern of human drug transporters. We have previously investigated the expression of ABC transporters in the human skin, revealing that a wide variety of ABC family transporters are expressed in the skin¹⁴. While SLC transporters play a role in the transport of a wide variety of endogenous and exogenous substrates, it has been recognized that 26 SLC transporters are critically involved in the transport of drugs⁶. Additionally, two SLC transporters, PCFT and RFC, play an important role in the transport of folic acid and reduced folate^{15,16}. In the present study, therefore, expression levels of these 28 SLC transporters, listed in Table 1, were determined in the human skin as well as in the liver and small intestine.

Acyl-glucuronide, a metabolite of carboxylic acid drugs, is a reactive metabolite that can bind cellular proteins and DNA to form protein- and DNA-adducts, which are associated with the development of adverse reactions. Ibuprofen, which is a nonsteroidal anti-inflammatory drug (NSAIDs) used for various health conditions, is metabolized by UDP-glucuronosyltransferases (UGTs) to produce ibuprofen acyl-glucuronide¹⁷. It has been reported that ibuprofen is associated with the onset of severe adverse reactions in the human skin, such as Stevens-Johnson syndrome¹⁸. In the present study, the effects of ibuprofen acyl-glucuronide on cell viability of human keratinocytes were also investigated. Expression microarray was applied to identify the causes of drug-induced adverse reaction in HaCaT cells.

Results

Validation of primers and PCR reactions. In the present study, the expression of SLC transporters and human peptide transporter (HPT) 1, which are associated with the transport of drugs, was examined in the human skin as well as in the liver and small intestine. Total RNA from human skin, the liver and small intestine was from three, one, and one individual donors, respectively. Primers used in the study were established with a program, Primer Blast (National Institutes of Health). We confirmed that all of the primer sets

Table 2 | Expression profiles of human SLC transporters in skin, liver, and small intestine

Subtypes	Skin 1	Skin 2	Skin 3	Liver	SI
SLC10A1				++++	
SLC10A2				++	
CDH17		+	+		+++
SLC15A1		+		++	++
SLC15A2				+	
SLC16A1	+	+	+++	++	+
SLC16A4	+	+	++++	+	++
SLC22A1				+++	
SLC22A2			++	+	
SLC22A3			+		+
SLC22A4			++	+	+
SLC22A5			++	++	+
SLC22A6					
SLC22A7				++	
SLC22A8	+				
SLC22A9	+	++		++	
SLC22A11	+	+			+
SLC28A3		++		++	++++
SLCO1A2				+++	
SLCO1B1		+		++++	
SLCO1B3	+	+		+++	
SLCO1C1			+		
SLCO2B1	+++	+++	++	+++	++++
SLCO3A1		++		++	
SLCO4A1	+++			++	+
SLCO4C1		++		+	
PCFT	+++	+++	+++	+++	+++
RFC		+	++	+	

Expression level of each SLC transporter is displayed by the number of +.

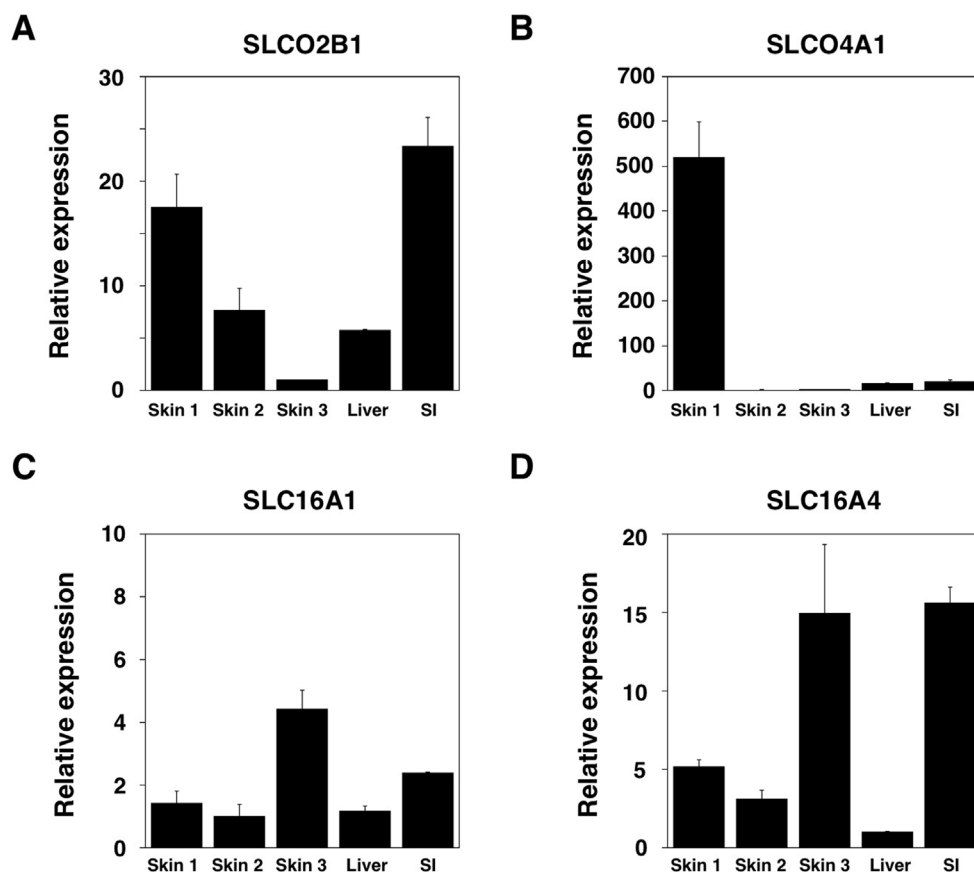


Figure 1 | Quantitative RT-PCR analysis of SLCO2B1, SLCO4A1, SLC16A1, and SLC16A4 mRNA in human tissues. Total RNA samples of human skin, liver, and small intestine were analyzed by RT-PCR using primers specific for each SLC transporter. Expression was normalized with the expression of GAPDH. SI, small intestine.

produced specific bands and that bands were not detected when the PCR reaction was conducted without cDNA. All of the PCR reactions were carried out with Ex Taq DNA polymerase (Takara).

Semi-quantitative RT-PCR. SLC10A1, SLC10A2, and cadherin (CDH)-17 genes encode Na⁺-taurocholate cotransporting polypeptide (NTCP), ileal bile acid transporter (IBAT), and HPT1, respectively. While those transporters are involved in the transport of endogenous substrates, such as bile acids and peptides, clinically used drugs can also be their substrates. Since NTCP is the main transporter that is responsible for the reabsorption of bile acids in hepatocytes, it has been reported that NTCP is highly expressed in the human liver¹⁹. In this study, it was demonstrated that the expression level of NTCP (SLC10A1) was significantly high in the human liver (Supplemental Fig. 1 and Table 2). Meanwhile, slight expressions of IBAT (SLC10A2) in the liver and HPT1 (CDH17) in the small intestine were observed. In the human skin, in contrast, none of these transporters were expressed, indicating that they have no role in the skin.

SLC15A1 and A2 genes encode peptide transporter (PEPT) 1 and PEPT2. As reported previously⁶, we observed a relatively higher and a slight expression of PEPT1 and PEPT2, respectively, in the liver and small intestine (Supplemental Fig. 1 and Table 2). Concentrative nucleoside transporter (CNT) 3 is encoded by the SLC28A3 gene. In contrast to a previous finding, we observed a higher and a moderate expression of CNT3 in the small intestine and in the liver, respectively. In the skin samples, none of these transporters were found to be expressed, except for a slight expression of CNT3 in one of three skin samples tested in this study.

SLC21 (SLCO) family genes encode various organic anion-transporting polypeptide (OATP) family transporters. It was demonstrated that OATP-C (OATP1B1) encoded by an SLCO1B1 gene was highly expressed in the liver⁶. In agreement with that report, a significant expression of OATP-C was observed in the liver in the present study (Supplemental Fig. 1). Other OATP family transporters were moderately expressed in the liver, while they were not expressed in the small intestine, except for OATP-B (OATP2B1), which is encoded by an SLCO2B1 gene. In the human skin, an expression of OATP-B was observed in all of the skin samples used in the present study, indicating that this transporter might play a physiological role in the skin in addition to regulating drug concentrations in skin cells. Although there was interindividual variability in the expression pattern of OATP family transporters in the skin, expressions of OATP-C, OATP8 (OATP1B3), OATP-F (OATP1C1), OATP-D (OATP3A1), OATP-E (OATP4A1), and OATP-H (OATP4C1), which are encoded by SLCO1B1, 1B3, 1C1, 3A1, 4A1, and 4C1 genes, respectively, were confirmed in the skin sample(s).

SLC16A1 and A4 genes encode monocarboxylate transporter (MCT) 1 and MCT2. These transporters were moderately or slightly expressed in the liver and small intestine (Supplemental Fig. 2 and Table 2), which is in agreement with the previous report⁶. Although the mRNA expression levels were different, these transporters were commonly expressed in all of the skin samples, suggesting that concentrations of their substrates in the skin cells could be different in each individual due to the large interindividual variability in the expression levels of those transporters in the skin.

SLC22 family genes encode organic cation transporters (OCTs), carnitine/organic cation transporters (OCTNs), and organic anion

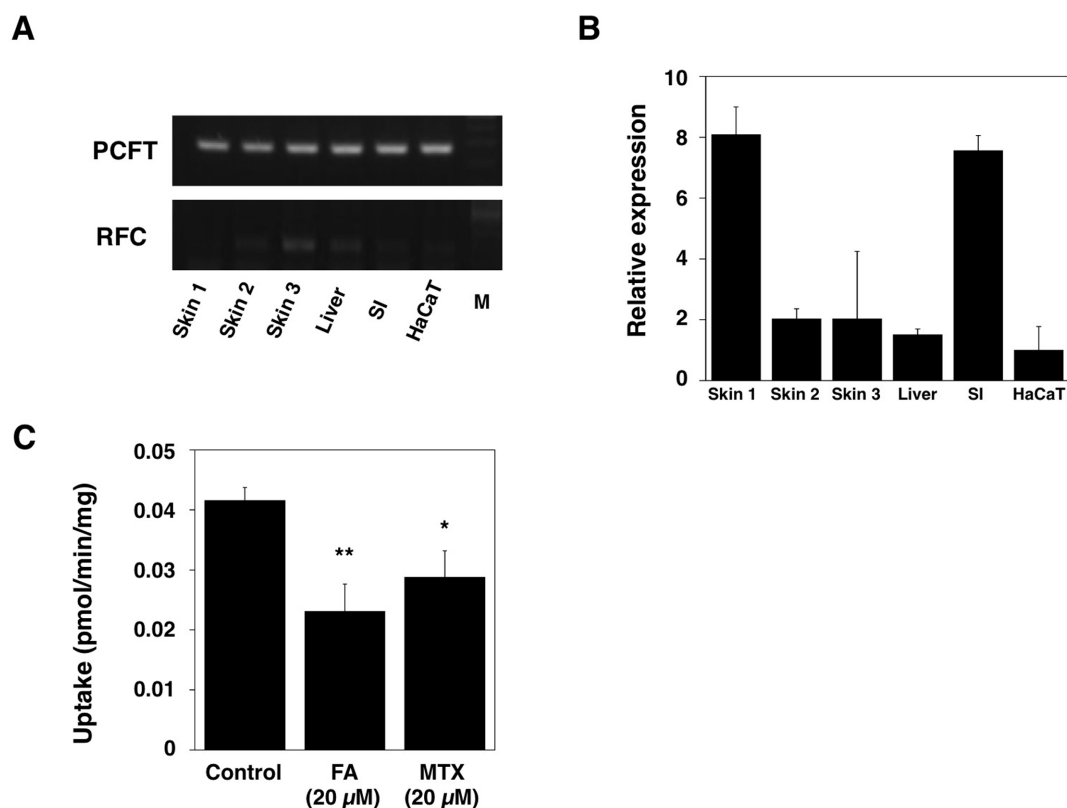


Figure 2 | Functional analysis of SLC transporters in human skin cells. (A), Total RNA samples of human skin, liver, small intestine, and HaCaT cells were analyzed by RT-PCR using primers specific for PCFT and RFC. SI, small intestine; M, 100 base pair marker. Full-length gels are shown in the supplementary information. (B), Quantitative RT-PCR analysis of PCFT in human skin, liver, small intestine, and HaCaT cells was conducted. Expression was normalized with the expression of GAPDH. (C), Uptake of FA into HaCaT cells was determined in the absence or presence of PCFT inhibitors. Column is the mean \pm S.D. of three independent determinations. *, $P < 0.05$; **, $P < 0.01$, compared with the transport activity in the control.

transporters (OATs). Because their primary role is renal excretion and reabsorption of organic cations and anions, these transporters are predominantly expressed in the kidneys, except for a high expression of OCT1, which is encoded by SLC22A1 in the liver⁶. Our data is in excellent agreement with the previous report (Supplemental Fig. 2 and Table 2). In the skin, the expression of most SLC22 family transporters was not detected; however, slight expressions of OCT2 (SLC22A2), OCT3 (SLC22A3), OCTN1 (SLC22A4), OCTN2 (SLC22A5), OAT3 (SLC22A8), OAT7 (SLC22A9), and OAT4 (SLC22A11) were observed in one skin sample.

Quantitative-RT PCR. Our semi-quantitative RT-PCR demonstrated that SLCO2B1, SLCO4A1, SLC16A1, and SLC16A4 were highly expressed in the human skin. To quantitatively analyze the expression, quantitative RT-PCR was conducted for these transporters. The expression pattern of the transporters in three skin samples as well as in the liver and small intestine was comparable between the quantitative- and semi-quantitative-RT-PCR (Fig. 1). The expression levels of SLCO2B1 and SLC16A1 in the skin were approximately the same as those in the liver. In contrast, the expression level of SLCO4A1 in one of the skin samples was more than 30-fold higher than that in the liver. It was also shown that the expression level of SLC16A4 in the skin was generally higher compared to that in the liver. These quantitative analyses indicated that there could be a wide interindividual variability in the expression of SLC transporters in the human skin.

Expression of PCFT and RFC. Our previous and current data indicate that a wide variety of ABC- and SLC-transporters are expressed in the human skin. This led us to further investigate the expression pattern of transporters involved in the transport of folate

and reduced folates. SLC46A1 and SLC19A1 encode proton-coupled folate transporter (PCFT) and reduced folate carrier (RFC), which transport folate and reduced folate, as well as clinical drugs such as methotrexate. Although an expression of RFC was not detected in the liver, small intestine, or skin, PCFT was highly expressed in all of these samples (Fig. 2A). Quantitative analyses also confirmed that the expression of PCFT in the human skin was comparable to that in the liver and small intestine (Fig. 2B). HaCaT cells have been derived from human keratinocytes²⁰. Prior to investigating the function of PCFT in HaCaT cells, it was confirmed that there was a high expression of PCFT in the cells, which was similar to the expression pattern observed in the human skin (Fig. 2A and B). Folate (Folic acid, FA) is one of the substrates of PCFT. In this study, uptake of FA into HaCaT cells was determined. The estimated uptake of FA into HaCaT cells was 0.041 ± 0.002 pmol/min/mg protein (Fig. 2C). It has been demonstrated that FA and methotrexate (MTX) are selective substrates of PCFT²¹. To investigate whether the uptake of FA into the cells was carrier-dependent or not, inhibitory effects of unlabeled FA or MTX on uptake of labeled ³H-FA into the HaCaT cells were examined. The uptake of ³H-FA was significantly inhibited by unlabeled FA or MTX (Fig. 2C), indicating that the substrate of PCFT accumulated in skin cells, possibly through carrier-mediated transport.

Effects of ibuprofen acyl-glucuronide and enalapril on cell viability and gene expression in HaCaT cells. SLC family transporters are associated with transport of drugs, as well as their metabolites, such as glucuronides, which can be responsible for drug-induced toxicity. In this study, when HaCaT cells were treated with ibuprofen acyl-glucuronide, concentration-dependent toxicity of acyl-glucuronide was observed as cell viability dropped to 80% and

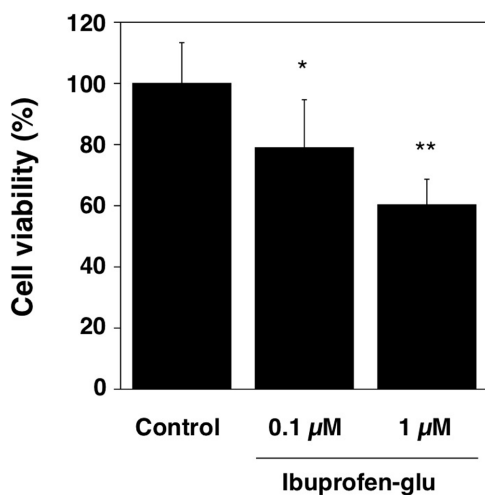


Figure 3 | Effects of ibuprofen-acyl-glucuronide on the cell viability. MTT assay was conducted to investigate the effects of ibuprofen-acyl-glucuronide on the cell viability of HaCaT cells. Each column is the mean \pm S.D. of three independent determinations. *, $P < 0.05$; **, $P < 0.01$, compared with the cell viability of the control cells.

60% when treated with 0.1 μM and 1.0 μM ibuprofen acyl-glucuronide, respectively (Fig. 3).

We further performed an expression microarray to determine the cause(s) of ibuprofen acyl-glucuronide-induced cytotoxicity. Total RNA was isolated from the control and ibuprofen acyl-glucuronide-treated HaCaT cells and was subjected to the microarray analysis, which contains 50,599 biological probes. The number of detected probes in the RNA from control and ibuprofen acyl-glucuronide-treated HaCaT cells was 27,318 and 27,285, respectively. Among the genes detected in both of the samples, 250 (115 up-regulated and 135 down-regulated) genes, including characterized and uncharacterized genes, changed more than 2-fold (Fig. 4 and Table 3). Table 4 shows the top 10 genes among the 250 that were up- or down-regulated more than 2-fold. Though as many as 23,281 genes were not detected in RNA from the control HaCaT cells, 218 genes were induced more than 5-fold in ibuprofen acyl-glucuronide-treated HaCaT cells, compared to their normalized signal values (Table 3). Similarly, while 23,313 genes were not detected in the RNA from ibuprofen acyl-glucuronide-treated HaCaT cells, 342 genes were reduced more than 5-fold in the control HaCaT cells, compared to their normalized signal values (Table 3). Table 5 shows the top 10 genes among the 560 genes that were induced or reduced more than 5-fold (218 + 342 genes). Among a number of genes whose expression levels significantly changed, S100 calcium binding protein A7A (S100A7A), which has been reported to be associated with drug-induced adverse reactions^{22,23}, was induced nearly 50-fold by the ibuprofen acyl-glucuronide treatment. Our microarray data was analyzed by Ingenuity Pathway Analysis (IPA) for signatures and pathway information. IPA analysis showed that most of the up- or down-regulated genes are involved in connective tissue disorders and cellular movement (Supplementary Table 1 and 2). It was further indicated that among numerous canonical pathways, methylglyoxal degradation I and bile acid biosynthesis-neutral pathway were the most positively- and negatively-affected by the chemical treatment (Supplementary Table 3 and 4).

To further investigate the effect of drug-induced cytotoxicity on gene expression, we treated the HaCaT cells with enalapril and performed MTT assays and quantitative RT-PCR. It has been reported that enalapril can induce severe skin disease such as psoriasis²⁴. As shown in Fig. 5A, concentration-dependent toxicity of enalapril was observed as cell viability dropped to 22% when treated with 100 μM enalapril. Expression of S100A7 was also induced by the treatment

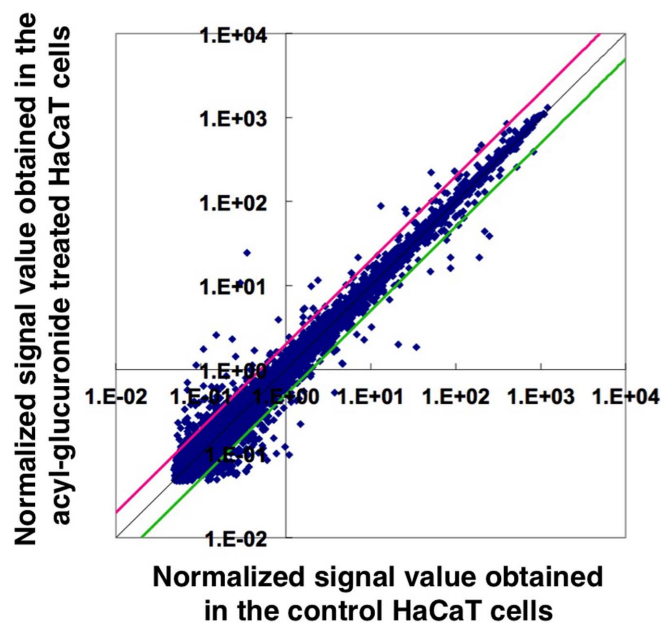


Figure 4 | Effects of ibuprofen-acyl-glucuronide on the cell viability and the gene expression in HaCaT cells. Microarray analysis was conducted to investigate the effects of ibuprofen-acyl-glucuronide on the gene expressions in HaCaT cells. Normalized signal value obtained in the control and acyl-glucuronide treated cells were shown.

with enalapril (Fig. 5B), indicating that S100A7 can be induced not only by active metabolites but also by a drug itself.

Discussion

Because primary antibodies specific to each SLC transporter are not fully available, protein expression levels of SLC transporters in human tissues are unable to be determined. On the other hand, mRNA expression levels of SLC transporters can be determined by RT-PCR with specific primers. Therefore, the mRNA expression pattern of human SLC transporters in the skin, liver, and small intestine were examined in this study. Previously, we analyzed the expression pattern of ABC transporters in human skin, finding that a wide variety of ABC transporters were highly or moderately expressed¹⁴. In contrast, our current study revealed that expression of SLC family transporters in human skin was restricted, while being highly expressed in the liver (Supplementary Fig. 1 and 2; Table 2). Similar to ABC transporters, there was significantly high interindividual variability in the expression levels of SLC transporters in human skin. This interindividual variability in the expression of transporters would cause substantial interindividual variability in concentrations of drugs and metabolites in skin cells, especially when the drugs and metabolites are substrates of SLC and ABC transporters expressed in the skin. This could result in a significant interin-

Table 3 | The number of genes increased or decreased by the treatment of HaCaT cells with ibuprofen acyl-glucuronide

Magunitude	More than 2-fold*	More than 5-fold**	Total
Increased	115	218	333
Decreased	135	342	477

*Among probes that were detected in both the samples (control and acyl-glucuronide treated HaCaT cells), the number of probes increased or decreased more than 2-fold was shown.

**Among probes that were detected in treated cells and not detected in control cells, the number of probes increased more than 5-fold was shown (Increased). Similarly, among probes that were detected in control cells and not detected in treated cells, the number of probes decreased more than 5-fold was shown (Decreased).



Table 4 | Top 10 probes that were up- or down-regulated more than 2-fold among the 250 genes

	Probe ID	Gene name	Genebank Accession	Fold change	
Increased	1	A_23_P142031	SH3-binding domain kinase family, member 2	NM_001101401	70.758
	2	A_23_P109133	arginine vasopressin	NM_000490	37.366
	3	A_33_P3341154	integrator complex subunit 1	NM_001080453	17.157
	4	A_24_P392925	glycolipid transfer protein domain containing 2	NM_001014985	16.282
	5	A_24_P66932	glycine cleavage system protein H (aminomethyl carrier) pseudogene	NR_033244	12.859
	6	A_33_P3337182			10.049
	7	A_21_P0010141			9.854
	8	A_33_P3404525	asialoglycoprotein receptor 1	AK308677	8.202
	9	A_21_P0013085			6.842
	10	A_23_P42322	collagen, type XI, alpha 2	NM_080680	5.976
Decreased	1	A_33_P3278033	tumor suppressor candidate 1	NM_001004125	0.054
	2	A_19_P00317016			0.088
	3	A_33_P3267482	mixed lineage kinase 4	NM_032435	0.095
	4	A_33_P3287661	uncharacterized LOC100130430	AK126997	0.114
	5	A_24_P67189	spermatid maturation 1	AK097400	0.122
	6	A_33_P3212350	caspase recruitment domain family, member 11	AK097139	0.130
	7	A_21_P0001579			0.140
	8	A_23_P204144	keratin 85	NM_002283	0.142
	9	A_33_P3214179			0.149
	10	A_23_P146885	urotensin 2 receptor	NM_018949	0.155

dividual variability in the probability of developing drug-induced skin diseases, as only certain patients develop adverse reactions, though the same amount of drugs is administered. Thus, determining the expression pattern of drug transporters prior to the administration of drugs might make it possible to avoid the onset of drug-induced skin diseases in individuals.

Although the skin is the primary tissue that protects our body from ultraviolet irradiation, infection, microorganisms, and exogenous toxic compounds, it is also one of the tissues where severe adverse reactions caused by administered drugs, such as Stevens–Johnson syndrome⁸, psoriasis⁹, allergy¹⁰, drug-induced hypersensitivity syndrome¹¹, Lyell syndrome¹², and drug-induced photosensitivity¹³, are seen. Because of the extensive usage of NSAIDs, a number of studies have been done and reports have shown the link between ingestion of NSAIDs and the onset of drug-induced cytotoxicity. Sternlieb and Robinson reported a patient who developed Stevens–Johnson syndrome after ibuprofen use in 1978¹⁸. Other drugs, such as methotrexate and carbamazepine, have also been reported to be associated

with the onset of Stevens–Johnson syndrome^{25,26}. *In vitro* studies indicated that those drugs are substrates of drug transporters such as SLC22A6 (ibuprofen and methotrexate), SLC22A7 (methotrexate), SLC22A8 (ibuprofen and methotrexate), SLC22A11 (ibuprofen and methotrexate), SLCO1B3 (methotrexate), PCFT (methotrexate), RFC (methotrexate), and ABC transporters (carbamazepine)^{27–31}. An important question raised was whether drug transporters expressed in the dermal cells were functional or not. To answer the question, we examined the uptake of FA into HaCaT cells, because FA is one of the substrates of PCFT, which was found to be highly expressed in the HaCaT cells (Fig. 2A). The estimated uptake of FA into HaCaT cells in our *in vitro* study was 0.041 pmol/min/mg (Fig. 2C), supporting our hypothesis that drug transporters in dermal cells are indeed functional and important for the regulation of cellular concentration of drugs.

The pathogenic mechanism of drug-induced skin diseases is not fully understood; however, recent studies have indicated that reactive metabolites generated by the metabolism of parent drugs

Table 5 | Top 10 probes that were up- or down-regulated more than 5-fold among the 560 genes

	Probe ID	Gene name	Genebank Accession	Fold change	
Increased	1	A_21_P0008561		BC038778	57.999
	2	A_19_P00323040			54.035
	3	A_33_P3393504	S100 calcium binding protein A7A	AY189119	46.420
	4	A_21_P0008711			39.687
	5	A_21_P0003052			32.507
	6	A_21_P0007698			30.895
	7	A_21_P0006088			29.528
	8	A_33_P3416867			26.352
	9	A_21_P0012166		BC039367	26.338
	10	A_33_P3219210			25.827
Decreased	1	A_33_P3328270	chromosome 9 open reading frame 172	NM_001080482	0.002
	2	A_21_P0010008			0.005
	3	A_33_P3301884	uncharacterized LOC116437	NR_026670	0.009
	4	A_32_P515971	EF-hand calcium binding domain 3	NM_173503	0.009
	5	A_33_P3325404	cysteine-rich protein 3	NM_206922	0.011
	6	A_33_P3346618	uncharacterized LOC441179	XR_112948	0.014
	7	A_33_P3341906	chromosome 10 open reading frame 128	BC037922	0.015
	8	A_21_P0002393		XR_108384	0.018
	9	A_21_P0002491			0.021
	10	A_21_P0006713			0.021

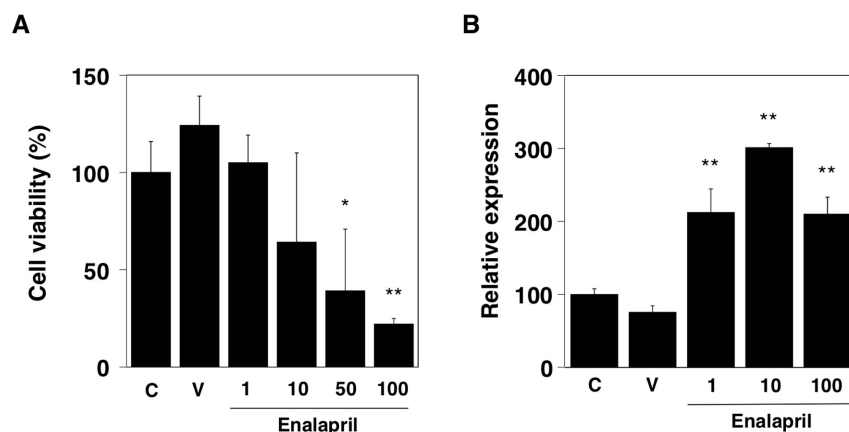


Figure 5 | Effects of enalapril on the gene expressions in HaCaT cells. (A), MTT assay was conducted to investigate the effects of enalapril on the cell viability of HaCaT cells. Each column is the mean \pm S.D. of four independent determinations. (B), Quantitative RT-PCR was conducted to investigate the effects of enalapril on the S100A7A (S100A15) expression in HaCaT cells. Expression was normalized with the expression of GAPDH. *, $P < 0.05$; **, $P < 0.01$, compared with the cell viability or the expression level in the control cells. C, control; V, vehicle treatment.

might be the main contributor to drug-induced adverse reactions. Troglitazone, flutamide, and acetaminophen caused significant decreases of cell viability in cytochrome P450 (CYP) 3A4-expressing cells, suggesting that reactive metabolite(s) produced by CYP3A4 might be involved in the cytotoxicity³², though the reactive metabolite(s) has not been identified. CYP2C9 has also been reported to be involved in the metabolic activation of a drug³³. In addition, formation of acyl-glucuronides of drugs with a carboxylic acid moiety through metabolism by UGTs have been linked to the development of hepatotoxicity³⁴. While those Phase I and II drug-metabolizing enzymes are mainly expressed in the liver, it was demonstrated recently that a wide variety of the enzymes are expressed in human skin³⁵; however, whether reactive metabolites could induce cytotoxicity in dermal cells or not has yet to be determined. In the current study, the cytotoxic effect of ibuprofen-acyl-glucuronide on HaCaT cells was analyzed. The observed concentration-dependent cytotoxic effect of ibuprofen-acyl-glucuronide (Fig. 3) indicated that not only parent drugs, but also their metabolites could be the cause of cytotoxicity in the skin. Although the pathogenic mechanism of acyl-glucuronide-induced cytotoxicity in the HaCaT cells is still unclear, our microarray study revealed that the expression of S100A7 was dramatically induced by treating HaCaT cells with ibuprofen acyl-glucuronide. This gene has been shown to be linked to keratinocyte differentiation and inflammatory skin disease^{22,23}. It has also been shown that an increase of S100A7 results in a release of various cytokines such as IL-6, IL-8, IL-17A, IL-22, and TNF- α , which are involved in inflammation and immune responses³⁶. Therefore, it was hypothesized that drugs and reactive metabolites such as acyl-glucuronide cause cytotoxicity by inducing inflammation and immune responses. Inhibition of the function of S100A7 in the skin might result in a suppression of the development of reactive metabolite-induced skin diseases. The involvement of other genes, listed in Table 4 and 5, in dermal cytotoxicity needs to be further determined to fully understand the underlying mechanism of metabolite-induced skin diseases.

In summary, this is the first study to identify the expression pattern of the family of human SLC transporters in the skin. The inter-individual variability in the expression levels of SLC transporters in human skin might be one of the determinants of developing drug-induced skin diseases. Determination of the expression of drug-metabolizing enzymes and transporters prior to administration of drugs would make it possible to avoid the development of idiosyncratic skin diseases in individuals.

Methods

Chemicals and reagents. The human total skin, liver, and small intestine RNA was purchased from Origene Technologies (Rockville, MD), Agilent Technologies (Santa Clara, CA), and Applied StemCell (Sunnyvale, CA). Primers were commercially synthesized at Invitrogen (Carlsbad, CA). Ex Taq DNA polymerase was purchased from TaKaRa Bio (Shiga, Japan). Ibuprofen acyl-glucuronide was purchased from Sigma (St. Louis, MO). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit was purchased from Nacalai Tesque (Kyoto, Japan). FA and MTX were purchased from Nacalai Tesque (Kyoto, Japan). [3 H]-FA (specific activity, 16.4 Ci/mmol) was purchased from Moravak Biochemicals, Inc. (Brea, CA). All other chemicals and solvents were of analytical grade or the highest grade commercially available.

Semi-quantitative reverse transcription (RT)-PCR. The complementary DNA (cDNA) was synthesized from total RNA using ReverTra Ace (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. The reverse-transcribed mixture was diluted 10-fold and a 1- μ l portion of the diluted solution was added to PCR mixtures (25 μ l). The amplification was performed by denaturation at 98°C for 10 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds for 40 cycles with Ex Taq DNA polymerase. The sequences of primers used in the present study are summarized in Table 1. The PCR products (20 μ l) were analyzed by electrophoresis with 2% agarose gel and visualized by ethidium bromide staining. Expression of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control for the cDNA quantity and quality.

Quantitative RT-PCR. Quantitative RT-PCR was performed with THUNDERBIRD SYBR qPCR Mix (Toyobo), and the reactions were run in a CFX96 Real-Time PCR Detection System (Bio-Rad). After an initial denaturation at 95°C for 30 s, amplification was performed by denaturation at 95°C for 5 s and annealing and extension at 60°C for 30 s for 45 cycles. The sequences of primers used in the quantitative RT-PCR study are as follows: S100A7A-forward: 5'-ACC TCG CCA CTG TCT TTG AG-3'; reverse: 5'-CCA TGG CTC TGC TTG TGG TA-3'; GAPDH-forward: 5'-CCA GGG CTG CTT TTA ACT C-3'; reverse: 5'-GCT CCC CCC TGC AAA TGA-3'. Primers for transporters used in the study are summarized in Table 1. Expression was normalized with the expression of GAPDH.

Cell culture and chemical treatments. HaCaT cells have differentiation characteristics similar to those of normal human keratinocytes³⁰. HaCaT cells were grown in Dulbecco's modified Eagle's medium supplemented with 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μ g/mL streptomycin, 0.1 mM nonessential amino acids (NEAA), 2 mM Gluta-MAXTM-I, and 10% fetal bovine serum with 5% CO₂ at 37°C. Before the chemical treatment, HaCaT cells (passage 40–45) were seeded into ninety six-well plates at 5×10^5 cells/well. After 48 hours, the culture medium was changed to a DMEM medium containing 0.1 to 1 μ M ibuprofen acyl-glucuronide or 1 to 100 μ M enalapril, and the cells were treated for 48 h. Ibuprofen acyl-glucuronide was dissolved in methanol with a final concentration in the medium of less than 0.1%. MTT assay was carried out according to manufacturers' instructions.

Expression array. Total RNA was isolated from control and ibuprofen acylglucuronide-treated HaCaT cells using TRIzol (Invitrogen). cRNA was prepared from the total RNA using the Quick Amp Labeling Kit (Agilent Technologies) following procedures recommended by the manufacturer. Briefly, 100 ng of the total RNA were reverse transcribed to cDNA followed by synthesis of cRNA incorporated



with cyanine 3 (Cy3)-labeled nucleotide. cRNA was then purified using RNeasy mini columns (Qiagen). Fluorescently labeled targets were hybridized to a SurePrint G3 Human GE 8 × 60 K DNA microarray containing 50,599 biological probes (Agilent Technologies). Hybridization and wash processes were performed according to the manufacturer's instructions, and hybridized microarrays were scanned using an Agilent Microarray Scanner (Agilent Technologies). Feature Extraction software (ver 10.7.1.1) was employed for image analysis and data extraction processes. Microarray data was further analyzed by IPA for signatures and pathway information (Ingenuity Systems, Qiagen, Hilden, Germany).

Statistical analyses. Statistical significances were determined by analysis of variance followed by Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

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Author contributions

R.F. and T.I. wrote the main manuscript text and S.T., M.H., T.N. and R.F. conducted experiments and prepared figures 1–5. All authors reviewed the manuscript.

Additional information

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