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Research Paper

Kinetic Analysis on the Skin Disposition of Cytotoxicity as an Index of Skin Irritation Produced by Cetylpyridinium Chloride: Comparison of *In Vitro* Data using a Three-Dimensional Cultured Human Skin Model with *In Vivo* Results in Hairless Mice

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Purpose. The aim of this study was to kinetically and dynamically analyze *in vitro* cytotoxicity as an index of skin irritation by use of a three-dimensional cultured human skin model and to compare the *in vitro* assay data with data from living animals.
Methods. A cationic surfactant, cetylpyridinium chloride (CPC), was selected as a model irritant. Living skin equivalent-high (LSE-high) and hairless mice were used for the *in vitro* and *in vivo* tests, respectively. Skin irritation dermatodynamics was evaluated by calorimetric thiazoyl blue (MTT) conversion assay both for *in vitro* and *in vivo* tests, whereas dermatokinetics of CPC in LSE-high and mouse skin were evaluated using HPLC.
Results. The time course of cell viability in the skin after application of CPC to intact skin was distinctly different from that of stratum-corneum-stripped skin in both LSE-high and hairless mice. Biphasic behavior characterized by two first-order rates with an inflection time point was observed in intact skin, whereas cell viability monoexponentially decreased immediately after CPC application in stripped skin. The time courses of cell viability in the skin and dermatodynamics were closely related to that of dermatokinetics of CPC.
Conclusion. The present study demonstrates that the *in vitro* cytotoxic profile was similar to the *in vivo* cytotoxicity test and that dermatodynamics was related to dermatokinetics of CPC.

KEY WORDS: Cetylpyridinium chloride; kinetics; living skin equivalent-high; MTT assay; skin irritation.

INTRODUCTION

It has recently been recognized that close attention must be paid to the use of pharmacokinetics/pharmacodynamics (PK/PD) to develop refined pharmaceuticals. The usefulness of PK/PD was first recognized by the FDA, and guidelines on the exposure–response relationships for drug development have been published (1). Many reports regarding the PK/PD correlation to plasma or serum concentration of a drug and its pharmacological effect have been published. Yet, few reports have discussed the PK/PD of nonsystemic drugs. However, for topical formulations with local effects, the relationship between drug concentration at the application sites and underlying tissues such as skin or subcutaneous tissues (not plasma or serum) (dermatokinetics) and its pharmacological effect (dermatodynamics) must be discussed. Moreover, kinetic and dynamic analyses of toxic compounds or irritants for side effects or skin irritation, also called toxicokinetics/toxicodynamics (TK/TD), as well as PK/PD relationships, are extremely useful for evaluation of topical formulations. Skin irritation is one of the most common cutaneous adverse effects, and evaluation of its potential and extent is indispensable in the development of topical formulations. Skin irritation is defined as reversible inflammation reactions (i.e., erythema and/or edema) that are produced by arachidonic acid cascades or cytokines of inflammatory mediators released from keratinocytes and fibroblasts as a result of exposure to chemicals or physical factors. Skin irritation has conventionally been evaluated *in vivo* by Draize tests in rabbits or guinea pigs (2) or directly by patch tests in humans. Recently, a three-dimensional cultured human skin model, with morphology and structure remarkably similar to real skin, was developed (3). In the present study, living skin equivalent-high (LSE-high, Toyobo Co., Ltd., Osaka, Japan) was used as a cultured human skin model. LSE-high is a membrane consisting of three-dimensional cultured cells, different from the monolayer membrane of epithelial cells such as Caco-2. This model is structurally constructed in two different layers, a multidifferentiated epidermal layer that consists of keratinocytes and a dermal layer that consists of fibroblasts embedded in collagen gel.

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ABBREVIATIONS: CPC, cetylpyridinium chloride; LSE-high, living skin equivalent-high; MTT, 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide.

The epidermal layer of this model has a basal layer, stratum spinosum, granular layer, and stratum corneum as well as living body skin (4,5). LSE-high is a skin model where the barrier function of the stratum corneum of LSE is improved by modifying the culture condition (6). The cultured human skin model was evaluated *in vitro* as an alternative for animal and human skin. However, a few problems remain for the cultured skin model: No erythema and edema were observed in the model, unlike in *in vivo* animal and human skin. To overcome this problem, a cytotoxicity test such as a 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or neutral red uptake was evaluated using the cultured skin model. Currently, this *in vitro* method has been validated (7) as an alternative for animal experiments by the European Centre for the Validation of Alternative Methods (ECVAM). Although relationships were reported between applied concentrations of test compounds (dermatokinetics) and skin irritation by the Draize test and MTT assay (dermatodynamics), few reports were found on the dermatokinetics/dermatodynamics relationship between the drug concentration in irritation sites (skin) and the skin irritation. Furthermore, the only end point evaluated was for the reported *in vitro* irritation studies (irritation was measured one or a few days after application of test samples). In addition, little or no reports were found on kinetic and dynamic analysis of skin irritation (time course of skin irritation).

In the present study, the time course of skin irritation determined by MTT assay and the relationship between skin irritation and the irritant concentration in the three-dimensional cultured human skin model as well as hairless mouse skin were analyzed using cetylpyridinium chloride (CPC) as a model test compound. LSE-high (Toyobo) was used as cultured human skin. CPC is widely used as a biocide in consumer products such as mouthwash (8) and is well known to be a skin irritant (9). In addition, the compound was used as one of the positive controls in validation of *in vitro* cytotoxicity test using cultured human skin model for evaluation of skin irritation in Japan (10,11). It has the advantage of being easily determined by HPLC-UV.

THEORETICAL

The kinetic and dynamic analysis of skin irritation was performed according to a previous procedure (12). In the previous report, and in this report, it was hypothesized that percent decrease in cell viability in the skin followed first-order kinetics after the skin irritant was applied to the skin surface. Moreover, we presumed that cell viability decreased immediately after the barrier function of the stratum corneum was damaged by the skin irritant. Thus, skin irritation kinetics in intact skin may be represented by two equations as follows:

$$V = (100 - V_\infty)\exp(-k_1 t) + V_\infty \qquad \text{when } 0 \le t < T \quad (1)$$

$$V = (100 - V_\infty)\exp(-k_1 T)\exp[-k_2(t - T)] + V_\infty \qquad \text{when } t \ge T \quad (2)$$

where V is percent cell viability in the skin at time t, T is the inflection time from the early slow to the late rapid decrease, and k_1 and k_2 ($k_1 \le k_2$) are first-order decreasing rate constants for the early and late phases, respectively. In addition, V_∞ is percent cell viability in the skin at infinite time after irritant application.

In contrast, cell viability in the skin after application to stratum-corneum-stripped LSE-high or stripped hairless mouse skin is shown as follows:

$$V = (100 - V_\infty)\exp(-k_{ss} t) + V_\infty \qquad \text{when } t \ge 0 \quad (3)$$

where k_{ss} is the first order decreasing rate constant on stripped skin.

MATERIALS AND METHODS

Chemicals

CPC was obtained from Tokyo Chemical Industry, Co., Ltd. (Tokyo, Japan). MTT was obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). All other chemicals and solvents used were of reagent grade or HPLC quality.

Animals and Skin Samples

Male Hos: Hr-1 hairless mice weighing 25–35 g were obtained from Hoshino Laboratory Animals (Kitakatsushika, Saitama, Japan). The animal experiment was approved by the Administration Committee at the Life Science Research Center, Josai University, and was carried out in accordance with its guidance. Skin was stripped by removing the stratum corneum with adhesive tape 20 times (13). LSE-high was obtained from Toyobo Co., Ltd. The stripped LSE-high was made in our laboratory by gentle removal of the stratum corneum with forceps.

CPC Application to Hairless Mice

The hairless mice were anesthetized by diethyl ether. A disc-shaped filter paper (No. 26-WA, Advantec MFS, Inc., Tokyo, Japan) with a diameter of 8.0 mm, containing 30 μL of 20% CPC solution in physiological saline, was applied either on the right or left abdominal skin with a Finn Chambers® on Scanpor® (8 mm, Epitest Ltd Oy, Tuusula, Finland). Physiological saline (0% CPC) alone was applied on the opposite side as a control. The animal's body was then wrapped with an elastic bandage (Elastpore®, Nichiban Co., Ltd., Tokyo, Japan) to prevent removal of the Finn Chambers®. At predetermined times, the application sites were carefully cleaned by gentle wipes of the skin with four moist pieces of cellulose cotton and excised for the MTT assay or measurement of CPC concentration in the skin.

CPC Application to LSE-High

One percent CPC in physiological saline (80 μL) was applied to the stratum corneum side for each period at 37°C and humidified atmosphere (95% O_2 and 5% CO_2). The CPC solution on the LSE-high was removed by a pipette and

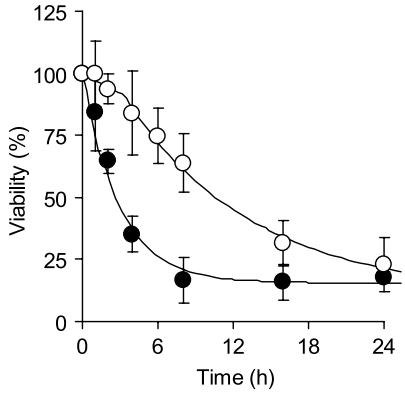

Fig. 1. Time course of the cell viability (%) of intact and stripped LSE-high after application of 1% CPC to LSE-high. (○) Intact skin, (●) stripped skin. Lines show theoretical values. Each value represents the mean ± SD (n = 3–7).

washed with the assay medium for LSE-high (Toyobo) and used for the MTT assay or measurement of CPC concentration in the tissue.

MTT Assay

The extent of skin irritation of LSE-high and hairless mouse skin was evaluated by the calorimetric MTT conversion assay (14). The metabolic reduction of this soluble tetrazolium salt to a blue formazan precipitate is dependent on the presence of viable cells with intact mitochondrial function (15).

MTT solution (1.2 mL) at a concentration of 0.333 mg/mL in the assay medium was then applied to the dermis side of the skin. After reaction for 3 h at 37°C in a humidified atmosphere, the tissue sample was washed with the assay medium. A tissue disc with a diameter of 8.0 mm was made by biopsy punch. The obtained tissue was transferred to a test tube and 1.0 mL of 0.04 N HCl in isopropanol was added to dissolve the formazan crystals. The absorbance of the solution was then measured at 570 nm using a spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan).

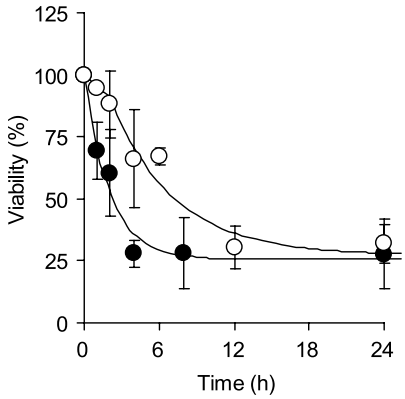

Fig. 2. Time course of the cell viability (%) of intact and stripped hairless mouse skin after topical application of 20% CPC to hairless mouse. (○) Intact skin, (●) stripped skin. Lines show theoretical values. Each value represents the mean ± SD (n = 3–7).

Table I. Kinetic Parameters for Decreased Cell Viability in LSE-high and Hairless Mouse Skin after Application of CPC

	LSE-high		Hairless mouse	
	Intact	Stripped	Intact	Stripped
CPC concentration (%)	1	1	20	20
k_1 (h^{-1})	0.032		0.091	
T (h)	3.21		2.38	
k_2 (h^{-1})	0.097		0.204	
k_{ss} (h^{-1})		0.296		0.494
V_∞ (%)	10.8	17.0	30.8	25.6

Separation of Epidermis and Dermis of the Skin Sample

The intact skin where CPC was applied was excised. The stratum corneum layers were removed by tape stripping (20 times) using an adhesive tape. Then the epidermis was separated by the following modified heating method (16). It was sandwiched between two sheets of aluminum foil and pressed on a glass plate for 60 s at 50°C in an incubator. Then the epidermis layer was peeled from the dermis with a dissection forceps. After weighing both layers of tissues, CPC concentration in each layer was measured with the following methods.

Measurement of CPC Concentration

Analysis of CPC was performed by use of HPLC. Samples of hairless mouse skin or LSE-high were minced using dissection scissors with acetonitrile containing nonyl p-hydroxybenzoate as an internal standard. Samples were then centrifuged (4°C, 13,000 × g, 10 min), and the supernatants were injected into HPLC. The HPLC system consisted of an autosampler (SIL-10ADvp, Shimadzu), a pump (LC-10ATvp, Shimadzu), a UV detector (SPD-10Avp, Shimadzu), a column oven (CTO-10ASvp, Shimadzu), and a reverse-phase C_{18} analytical column (5 μm, 4.6 × 250 mm, Inertsil ODS-2, GL Science, Inc., Tokyo, Japan). Acetonitrile/0.2% sodium perchlorate (80:20) was used as mobile phase. The flow rate was 1 mL/min, and the column temperature was maintained at 40°C. The CPC content of the samples was analyzed using the UV detector set at 258 nm.

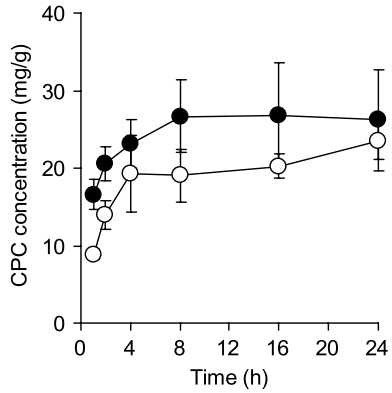

Fig. 3. Time course of skin concentration of CPC after application of 1% CPC to LSE-high. (○) Intact skin, (●) stripped skin. Each value represents the mean ± SD (n = 3).

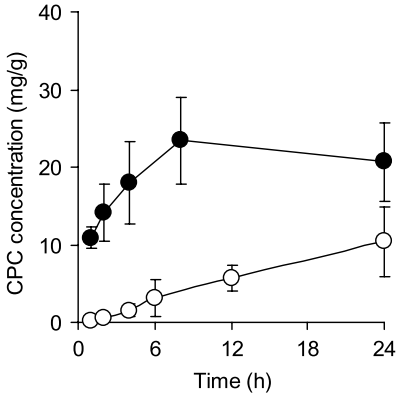

Fig. 4. Time course of skin concentration of CPC after topical application of 20% CPC to hairless mouse. (○) Intact skin, (●) stripped skin. Each value represents the mean ± SD (n = 3).

Histochemical Analysis for Succinate Dehydrogenase

The dissected skin was embedded in embedding material (O.C.T. compound, Tissue-Tek® 4583, Sakura Finetechnical Co., Ltd., Tokyo, Japan), frozen in 2-methylbutane cooled with dry ice, and cut in 10-μm sections with a cryostat (CM3050S, Leica, Nussloch, Germany). The sections were attached to a microscopic glass slide. Succinate dehydrogenase activity was determined by incubating the specimens for 60 min at 37°C in the dark with 500 mg of sodium succinate, 50 mg of nitroblue tetrazolium (NBT), and 1 mg of phenazine methosulfate dissolved in 100 mL of 0.1 M Tris–HCl buffer (pH 7.4). Then the specimens were quickly and sequentially dipped into aqueous acetone solutions with different concentrations in the order 30, 60, 90, 60, and 30%. Finally, the specimens were enclosed in Aquatex® (Merck KGaA, Darmstadt, Germany) after thorough washing with distilled water and immediately examined microscopically.

RESULTS

Figure 1 shows the time course of cell viability in the skin (*in vitro* data) after application of 1% CPC to intact (full thickness) LSE-high and stratum-corneum-stripped LSE-high. The time course of cell viability in intact LSE-high was markedly different from that in stripped LSE-high. In particular, biphasic behavior with an inflection point was observed in the intact LSE-high. Figure 2 shows the time course of cell viability in the skin (*in vivo* data) after topical application of 20% CPC to intact skin and stripped skin of hairless mouse abdomen. The *in vivo* time course of cell viability in intact skin was different from that in stripped skin in hairless mice. These *in vitro* and *in vivo* time courses of cell viability in the skin were kinetically analyzed using Eqs. (1)–(3), as explained in the Theoretical section. The obtained values for each parameter are shown in Table I. The parameters, k_1, T, k_2, and V_∞ were calculated to be 0.032 h^{-1}, 3.21 h, 0.097 h^{-1}, and 10.8%, respectively, in the intact LSE-high, whereas k_{ss} and V_∞ were 0.296 h^{-1} and 17.0% in the stripped LSE-high. On the other hand, k_1, T, k_2, and V_∞ were 0.091 h^{-1}, 2.38 h, 0.204 h^{-1}, and 30.8%, respectively, in intact skin of hairless mice, whereas k_{ss} and V_∞ were 0.494 h^{-1} and 25.6% in stripped skin of hairless mice. We hypothesized that the higher first-order rate constant after the inflection point in intact skin, k_2, must be very close to the rate constant in stripped skin, k_{ss}. Unfortunately, this expectation was not correct in both LSE-high and hairless mice. The existence of an inflection point in intact skin is closely related to a sudden decrease of barrier function of the stratum corneum. Because value of k_{ss} was twice to thrice that of k_2 in both LSE-high and hairless mouse, destruction of the barrier function of the stratum corneum by CPC may not be complete. Furthermore, species differences in cytotoxicity were observed in both k_2 and k_{ss}, and these parameters in mouse skin were approximately twice those in LSE-high.

The time courses of skin concentration of CPC after application to LSE-high or hairless mouse are shown in Figs. 3 and 4. CPC concentration in intact LSE-high became almost constant 4 h after starting the experiments. CPC concentration in hairless mouse skin showed a tendency to maintain a steady-state level. However, the time was later than that in LSE-high and a discrepancy was observed between both time courses of cell viability in the skin and CPC concentration in hairless mice. The decrease in cell viability in the skin could be expressed in the first-order rate in hairless mice as well as in LSE-high. Consequently, it was

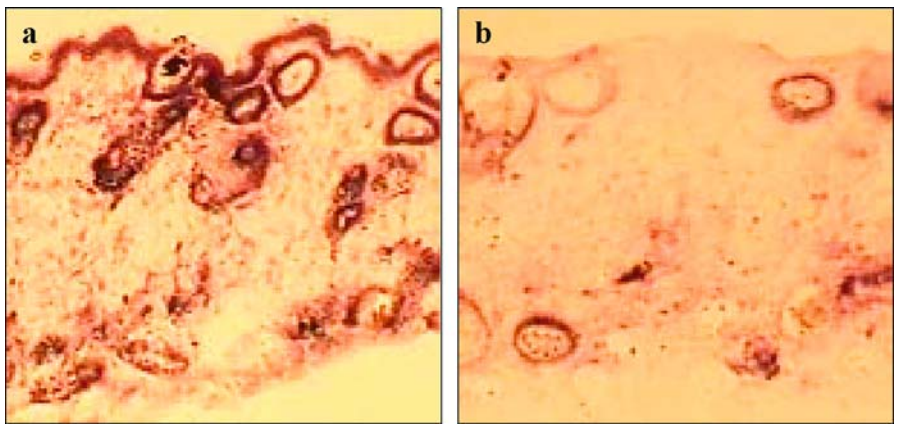

Fig. 5. Enzyme histochemical analysis for succinate dehydrogenase activity in mouse skin. An application site of (a) physiological saline as control, (b) 20% CPC for 24 h. Magnification ×10.

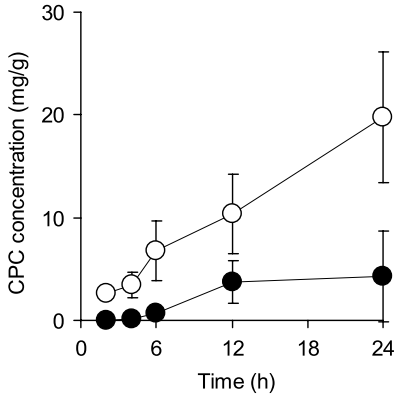

Fig. 6. Time course of CPC concentration in epidermis and dermis after topical application of 20% CPC to hairless mouse. (○) Epidermis, (●) dermis. Each value represents the mean ± SD ($n = 3$).

considered that a constant decrease of cell viability in the skin can be obtained above a certain level of CPC in skin.

Although all skin layers were used for the present MTT assay, skin irritation may take place mainly in the upper layer (the outer layer) of skin (i.e., epidermis). Thus, frozen sections of the mouse skin were prepared after application of CPC and analyzed for succinate dehydrogenase. Succinate dehydrogenase is a marker enzyme of mitochondria, and the principles of the enzyme assay are the same as those of the MTT assay. The results are shown in Fig. 5. The bluish-purple color for formazan was observed throughout the whole skin, and the color was paler in skin on which CPC was applied as compared to control skin. In addition, the upper skin (epidermis) had a tendency to show a high level of the enzyme. We then separated the epidermis from the dermis of hairless mouse skin onto which CPC was applied and measured the CPC concentration in each layer. The results are shown in Fig. 6. In the experiment, the stratum corneum was removed by tape stripping. The CPC concentration in the dermis showed a tendency to reach a constant level after 12 h. In contrast, no steady-state level in the CPC concentration was obtained in the epidermis: The CPC level in the epidermis markedly increased after four hours.

DISCUSSION

Only a few reports have been published on toxicokinetic/toxicodynamic (TK/TD) relationships. In addition, fewer reports have been published on dermatokinetic/dermatodynamic relationships. Theoretical aspects of TK/TD and dermatokinetic/dermatodynamic relationships, however, are similar to PK/PD relationships. Relationships between the effect or toxicity and dose of a drug can be categorized as a "direct reaction model," wherein a drug directly acts at the action site, or as an "indirect reaction model," wherein a drug enhances or inhibits biological reaction independent of the drug concentration (17,18). Skin sensitization among dermal adverse reactions may obey an indirect reaction model. Irritation and cytotoxicity are observed when an irritant, such as keratinocytes or fibroblasts, is added to cultured cells (19). The obtained relationship between the applied irritant concentration and the reaction may be expressed in the direct reaction model. Thus, the primary skin irritation can be expressed mostly by the E_{max} model in direct reaction models (20).

The reaction site(s) for skin irritation are epidermal keratinocytes and dermal fibroblasts. Thus, an irritant must penetrate into the stratum corneum, the biggest barrier of skin permeation, to cause skin irritation. We assumed that cell viability in the skin decreases by a first-order reaction, when the irritant level at the reaction sites is constant or above a certain level.

In the present study, the time course of cell viability in the skin by MTT assay showed a biphasic profile in both intact skin of LSE-high and hairless mice. These results corresponded to our previous results using lactic acid and sodium lauryl sulfate (12). CPC penetration is low and, thus, CPC concentration in the skin is low immediately after the irritant application because the barrier function of the stratum corneum is sufficient. The low concentration of the irritant in skin is related to the low decreasing rate of cell viability in the skin. A few hours later, the skin concentration increased, with disruption of the stratum corneum barrier, and the decreasing rate of cell viability in the skin increased. On the other hand, cell viability in the skin monoexponentially decreased immediately after CPC application in the stripped skin both for LSE-high and hairless mice. This result suggests there was almost no delay (lag time) of this reaction by CPC. The CPC concentration in skin when cell viability in the skin became constant was believed to change as follows. The cell viability in stripped skin decreased immediately after CPC application, and the cytotoxic level in the skin was considered to be less than 10 mg/g. In contrast, an inflection point was obtained after application to the intact skin, and the irritation-produced level in skin was more than 1.5 mg/g.

Skin irritation by chemicals applied to the skin can be determined by the product of the potential of a molar unit of a chemical and its concentration at the reaction site. It is well known that the stratum corneum is the primary skin barrier against skin permeation or penetration (21). The presence of the stratum corneum itself is the cause of discrepancy between actual skin irritation and irritation potential of the chemicals, and complicates the evaluation of skin irritation using simple cultured cells.

Similar skin irritation behavior of LSE-high (*in vitro*) and hairless mouse (*in vivo*) observed in the present study suggests the usefulness of an evaluation method for skin irritation using a three-dimensional human cultured skin model. However, the concentration of CPC applied to LSE-high was about 1/20th of that applied to hairless mice in this experiment. Furthermore, the CPC concentration in intact LSE-high was higher than that in intact hairless mouse skin, but that in stripped LSE-high was almost the same as that in mice. Thus, the CPC concentration showing similar cytotoxicity in LSE-high must be almost the same as that in mice. The dose showing similar toxicity was calculated to be 20 times different between LSE-high and mice. Because CPC is a hydrophilic compound, it was considered from the difference in the CPC concentrations in intact skin that the barrier function of the stratum corneum was markedly influenced. In other words, this difference was due to the difference in the skin permeability of CPC. It is known that permeability of a drug in LSE-high is generally higher than that through real

human skin. We have already studied skin permeability using cultured skin and real human and animal skins (22). No significant difference was observed in the partition parameters of several drugs between LSE-high and animal or human cadaver skin. However, the diffusion parameter was different among the skins, and the cumulative amount of several drugs that permeated through LSE-high was about 10-fold higher than those through human cadaver skin. This suggests that the permeability difference between LSE-high and the human cadaver skin may be attributed to diffusion rate. Currently, various companies provide reconstituted *in vitro* human skin equivalents such as Epiderm (MatTek, Ashland, MA, USA), Episkin (Episkin, Chaponost, France), Apligraf (Organogenesis Inc., MA, USA), and Skinethic (Skinethic, Nice, France). These cultured human skin models are epidermis models. By contrast, LSE-high has both the epidermis layer and the dermis layer. Although no direct comparative data were found, permeability of a drug through LSE-high may be different from these epidermis models. In any case, because permeation of a drug in LSE-high is more rapid than in human skin, a skin irritation assay using cultured skin may be overestimated against irritation in real skin.

Therefore, problems remain for estimation of skin irritation and related irritant concentration, although examination for the rank order of skin irritation is not a significant issue. One has to pay attention to the existence/absence of skin irritation at a certain dose. Therefore, the relationship was usually estimated between the applied concentration of a test compound and resulting skin irritation after sufficient time has passed. It was clarified in the present study that time courses of the relationship between the drug concentration and irritation in the reaction site can be kinetically evaluated.

Skin irritation is defined as an inflammatory reaction produced by inflammatory mediators that are released from keratinocytes and fibroblasts when skin is damaged by a chemical or physical factor. Furthermore, the inflammatory cells which exude from blood are also considered to participate in the skin irritation. Skin irritation causes skin erythema and edema.

In the present study, we evaluated CPC-produced skin irritation by cytotoxicity using the MTT assay. This method using a cultured human skin model has been validated by ECVAM. The prevalidation results showed that some are false-negative and false-positive among 20 chemicals (10 irritants and 10 nonirritants) and that specificity of the assay result was still only 67%, suggesting that measuring cytotoxicity does not always reveal the correct prediction between irritants and nonirritants (7,23). Skin irritation is complex, so there are various kinds of skin irritation with different mechanisms. In addition, it is well known that there are irritants that produce relatively low levels of cytokine, such as sodium lauryl sulfate (SLS), and irritants that produce high levels of cytokine, such as croton oil (24). SLS is an anionic surfactant and one of the most famous skin irritants. CPC, a model compound in the present study, is a cationic surfactant and has been found to be a typical skin irritant (9). Many ionic (anionic and cationic) surfactants were found to decrease the barrier function of the stratum corneum (25), as observed in the present study. This is known as a primary mechanism for the enhancement effect of skin permeation by surfactants. Ionic surfactants especially swell the stratum corneum and interact with intercellular keratin (26). Moreover, ionic surfactants disrupt cell membranes, resulting in the release of cytoplasm (27,28). Thus, skin irritation of the surfactants can be evaluated using cytotoxicity tests.

An *in vitro* skin irritation test measuring cytotoxicity is proposed as an alternative to animal experiments and has been examined by many researchers (29,30). On the other hand, there is discussion as to whether skin irritation is the same as cytotoxicity. Furthermore, the *in vivo* cytotoxic profile of an irritant was not clear until now. It became clear from the present study that a similar cytotoxic profile was observed for the *in vivo* and *in vitro* cytotoxicity tests. Because the *in vitro* cytotoxicity test using cultured human skin model is broadly used as an alternative method to evaluate skin irritation in animals, kinetic analysis of the *in vitro* cytotoxicity must be important as a new tool for evaluation of skin irritation, and the *in vitro–in vivo* correlation for skin irritation is also important for an in-depth understanding of skin irritation. The present study demonstrates results only for CPC, a cationic surfactant. Moreover, the hairless mouse was used for the *in vivo* model. Drug permeability through mouse skin must be different from that through human skin. However, because *in vivo* cytotoxicity test using humans is unfeasible, animals had to be used as the *in vivo* model in this study. In this type of study, the species difference, including human, in skin irritation is an important issue for numerous compounds. Cytotoxicity tests using the cultured cell may be useful for evaluation of mouse–human correlation of cytotoxicity. They are under way in our laboratory.

With regard to skin irritation as an inflammation reaction, it is easy to understand that cytokine release is one of the important mechanisms. Cytoplasm of keratinocytes in the epidermis contains interleukin-1α, which is a proinflammatory cytokine, a so-called "alarm cytokine" (31). Released interleukin-1α induces production of inflammatory cytokines including itself, and a more complicated inflammatory cascade (cytokine network) is formed (32,33). We will now also evaluate the time course of inflammatory mediators released and concentrations of irritants in the skin.

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