Utility of MTT Assay in Three-Dimensional Cultured Human Skin Model as an Alternative for Draize Skin Irritation Test: Approach Using Diffusion Law of Irritant in Skin and Toxicokinetics-Toxicodynamics Correlation

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Purpose. A cytotoxicity assay using a three-dimensional cultured human skin model, Living Skin Equivalent-high (LSE-high) was evaluated as an alternative to the Draize skin irritation tests using animals. A relation between the cytotoxicity and calculated concentration of an irritant in skin was also evaluated.

Methods. Colorimetric thiazoyl blue (MTT) conversion assay and a surfactant, cetylpyridinium chloride (CPC), were selected as a cytotoxicity assay and a model irritant. The fraction of dead cell number in the MTT assay or the Draize irritation score (*in vitro* and *in vivo* irritation data, respectively) was treated as a function of CPC concentration in the viable skin of LSE-high and guinea pig. Separately, *in vitro* permeations of CPC through the LSE-high or excised guinea pig skin were determined to calculate the average concentration of CPC in the viable skin using the Fickian diffusion theory. The obtained relations between the irritation scores and CPC concentration were evaluated by the Emax model (Hill equation).

Results. CPC concentration showing 50% irritation (IC_{50}) was similar for the MTT assay (18.9%) and Draize test (12.3%), and a good relationship ($r = 0.981$) was observed between the fraction of dead cell number and the Draize score. In contrast, IC_{50}, 1.32%, for the MTT assay in LSE-high was much lower than that using guinea pig skin. We then corrected the results for the MTT assay using a ratio of IC_{50} in guinea pig skin against LSE-high, resulting in a good relation between both MTT results in guinea pig skin and LSE-high.

Conclusion. The present results suggest that the MTT assay using LSE-high may be utilized as an alternative for the Draize test in animals for evaluating skin irritation.

KEY WORDS: Cetylpyridinium chloride; Draize score; Living Skin Equivalent-high; MTT assay; skin irritation.

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ABBREVIATIONS: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CPC, cetylpyridinium chloride; LSE-high, Living Skin Equivalent-high.

INTRODUCTION

Nowadays human beings are exposed to several kinds of chemicals in our daily lives. Among them, we use cosmetics directly on our skin almost everyday. Much attention has thus been paid to safety and skin irritation of cosmetics. Skin is also one of the administration sites of pharmaceutical formulations. Not only topical dosage forms expecting local pharmaceutical effects on the application site and its surroundings, but also transdermal therapeutic systems (TTS) of which the pharmaceutical effects act after absorption of the active ingredients into the systemic circulation are on been in the market (1). In addition, many trials have been reported to increase the drug penetration into the skin barrier, stratum corneum, i.e., penetration enhancers (2), iontophoresis (3), and electroporation (4). Application of active ingredients and pharmaceutical additives may cause skin irritation and further increase in the skin irritation is caused by chemical enhancers and electric current. Thus, skin irritation is a very important issue to rapidly assay and evaluate.

Skin irritation is defined as a reversible inflammatory reaction produced by the arachidonic acid cascade and cytokines in the viable skin cells like keratinocytes and fibroblasts (5). Inflammatory cells, which are penetrated by the fibroblasts and blood vessels, also relate to skin irritation, resulting in redness and edema in the viable epidermis and dermis (6). The Draize skin irritation test has been applied to evaluate cosmetic, pharmaceutical, and environmental chemical substances (7). There are still some scientific problems for extrapolation of the animal (rabbits and guinea pigs) data to human profiles. In addition, the ethical problems of animal experiments are more recognized than before. We thus need an alternative for the Draize irritation test to estimate the irritation problems in humans (8).

On the other hand, tissue culture technology has developed three-dimensional cultured human skin models (9). As the cultured models have no vasculature or migrating inflammatory cells, ultimate manifestations *in vivo* of edema and erythema cannot be simulated in these systems (10). Fortunately, however, inflammatory mediators like IL-1α, IL-8, and PGE_2 have already been found (11–13). The extent of skin irritation has been evaluated by the amount of inflammatory mediator released from viable skin cells by the colorimetric thiazoyl blue (MTT) conversion assay and the neutral red (NR) assay using cultured cell membranes (14,15).

In vitro and *in vivo* irritation scores in these tests (i.e., the MTT assay and Draize test) were generally related to the application amount or concentration of irritants on the skin. In contrast, the extent of the skin irritation must be directly related to the irritant concentration at the irritation site of the skin, i.e., viable epidermis and dermis according to the concept of toxicokinetics and toxicodynamics. Colorimetric MTT conversion assay and a surfactant, cetylpyridinium chloride (CPC), were selected as a cytotoxicity assay and a model irritant. CPC was selected due to easy determination of skin permeation. Living Skin Equivalent-high (LSE-high) was used for three-dimensional cultured human skin. LSE-high is an organotypic co-culture composed of human dermal fibroblasts in a collagen-containing matrix overlaid with human keratinocytes that have formed a stratified epidermis. *In vitro* permeations of CPC through the LSE-high or excised guinea

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pig skin were determined to calculate the average concentration of CPC in the viable skin using the Fickian diffusion theory for the penetrant diffusivity in the matrix. This calculation was done instead of direct measurement of CPC concentration in skin. The Draize test was done in guinea pigs and the MTT assay was performed in guinea pig skin and LSE-high. Stratum corneum-stripped skin was used, as well as the full-thickness skin, as a model having a lower barrier function against the irritant. The Emax model (Hill equation) was used to evaluate the obtained relations for both skins between the observed irritation scores and CPC concentration in the viable epidermis and dermis. The Hill equation is a typical approach to express the relationship between toxicokinetics and toxicodynamics.

THEORETICAL

The extent of skin irritation is generally dependent on the amount of applied irritant on the skin. The extent of skin irritation, however, is more directly dependent on the irritant concentration at the irritation site of the skin. The irritation site must be in the viable epidermis and dermis not in the stratum corneum, the uppermost layer of skin. Thus, irritant concentration in the viable skin tissue is a good index for the skin irritation. It is very difficult, however, to directly measure the concentration of irritant such as surfactants in the viable epidermis and dermis. In the present study, the irritant (CPC) concentration in the viable skin was determined by calculation using skin permeation data of the irritant and the Fickian diffusion theory of the penetrants in the skin membrane instead of direct measurement.

The calculation method of the irritant concentration in the viable epidermis and dermis was as follows. When an irritant was applied on the stratum corneum-stripped skin (as a low barrier skin model), the average concentration of the irritant in the viable epidermis and dermis, C, can be derived by the following equation according to Fick's law of diffusion.

$$C = \frac{K_{ved}}{2} \cdot C_v \tag{1}$$

where C_v is the irritant concentration in the applied vehicle and K_{ved} is the partition coefficient of the irritant from the vehicle to the viable epidermis and dermis. In contrast, when the irritant is applied on the intact (full-thickness) skin, C becomes

$$C = RR \cdot \frac{K_{ved}}{2} \cdot C_v \tag{2}$$

where RR is the ratio of diffusion resistance in the viable epidermis and dermis against that in the full-thickness skin. The RR can be expressed as

$$RR = \frac{P_{sc}}{P_{sc} + P_{ved}} \tag{3}$$

where P_{sc} and P_{ved} are the permeability coefficients of the irritant in the stratum corneum and viable epidermis and dermis, respectively. The permeation resistance of the irritant through a membrane is reciprocal to the permeability coefficient.

As shown above, K_{ved} is necessary to determine the C value from the application concentration C_v. K_{ved} was obtained from the skin permeation profile as follows. Two assumptions can be made in the present *in vitro* skin permeation experiment: a sink condition is established in the receiver compartment of the diffusion cell throughout the experiment, and viable epidermis and dermis is a homogeneous single membrane in addition to the stratum corneum, which is another homogenous membrane. Thus, the time course of change in the cumulative amount of the irritant, which has permeated through the viable epidermis and dermis, Q, against time, t, can be expressed by the following equation

$$Q = K_{ved} \cdot L_{ved} \cdot C_v \left[\frac{D_{ved}}{L_{ved}^2} \cdot t - \frac{1}{6} - \frac{2}{\pi^2} \sum_{n=1}^{\infty} \frac{(-1)^n}{n^2} \exp \left(- \frac{D_{ved}}{L_{ved}^2} \cdot n^2 \cdot \pi^2 \cdot t \right) \right] \tag{4}$$

where D_{ved} is the diffusion coefficient of the penetrant in the viable epidermis and dermis and L_{ved} is the thickness of the viable epidermis and dermis. The partition parameter ($K_{ved} \cdot L_{ved}$) was obtained by curve fitting of the permeation data to Eq. (4) using the nonlinear least square method. In this calculation, 530 and 1130 μm was applied for the thickness of the viable epidermis and dermis of LSE-high and guinea pig skin, respectively (16).

On the other hand, a relationship between the extent of skin irritation and the irritant concentration at the irritation site can be evaluated by the Emax model (Hill equation) as follows according to a theory for toxicokinetics/toxicodynamics

$$I = \frac{I_{\max} \cdot C^{\gamma}}{IC_{50}^{\gamma} + C^{\gamma}} \tag{5}$$

where I is the extent of skin irritation, $I_{\max}$ is the maximum extent of skin irritation, C is the irritant concentration at the irritation site, IC_{50} is the irritant concentration at the irritation site showing 50% of skin irritation against $I_{\max}$, and γ is a shape-determining factor.

In this experiment, magnitude scale of Draize score was treated as a continuous quality after calculating average value according to previous works (17,18). Similar treatment of the magnitude scale was also reported for mood score after administration of opioids in patients (19).

MATERIALS AND METHODS

Materials

CPC was obtained from Tokyo Chemical Industry, Co., Ltd. (Tokyo, Japan). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was obtained from Sigma Chemical Co. Ltd. (St. Louis, Missouri). All other chemicals and solvents used were of reagent grade or HPLC quality.

Animals and Skin Samples

Male Hartley guinea pigs weighing between 300–400 g were obtained from Saitama Experimental Animal Laboratory (Sugito, Saitama, Japan) and used for all the animal experiments. The experiments were carried out according to the guidance of the Life Science Research Center, Josai University. A guinea pig was fixed on his back and the abdominal skin was treated using a hair clipper and a shaver to remove

the hair. Stripped skin was achieved by stripping the stratum corneum off with adhesive tape 20 times (20). These guinea pigs were kept in a cage for 24 h before the experiment.

The abdominal guinea pig skin was excised for the CPC permeation experiments. Full-thickness LSE-high (Tsuruga Institute of Biotechnology, Toyobo Co., Ltd., Tsuruga, Fukui, Japan) or stratum corneum-stripped LSE-high were also used for the permeation experiments. The stratum corneum-stripped LSE-high was made in our laboratory using forceps as shown in our previous report (21).

Skin Permeation Study

Skin permeation study was carried out under consideration of a previously established method (22). The obtained intact guinea pig skin, stripped guinea pig skin, LSE-high or stripped LSE-high was mounted in a two-chamber (side-by-side) diffusion cell (effective diffusion area: 0.95 cm^2)(23). CPC (applied concentration; 5.0% for guinea pig skin and 1.0% for LSE-high) in physiological saline (2.5 mL) was applied to the epidermal side, and the same volume of physiological saline to the dermis side. The lower concentration of CPC against the LSE-high was so as not to damage the skin by the surfactant. Skin permeation of CPC was followed at 37°C by periodical sampling of 1.0 mL from the receiver solution, and then the same volume of the same saline solution was added to keep the volume constant. The drug concentration in each sample was assayed by HPLC to determine the cumulative amount of CPC that permeated through the skin at each sampling time point.

HPLC Analysis

CPC was determined by HPLC. The mobile phase used was pH 3.5 phosphate buffer containing 5 μmol/mL sodium 1-heptanesulfonate: acetonitrile (70:30). The receiver sample was added to acetonitrile solution containing hexyl *p*-hydroxybenzoate as an internal standard. Each sample was injected into an HPLC system composed of a pump (LC-$10AT_{VP}$), a detector (SPD-$10AT_{VP}$), an integrator (SIL-$10D_{VP}$) (those from Shimadzu, Kyoto, Japan), and a reverse-phase column (Inertsil ODS-2, 4.6 × 250 mm, GL Science, Inc., Tokyo, Japan). The flow rate was 1.0 mL/min, and the column temperature was maintained at 40°C. The CPC detection was done using a UV detector with a wavelength of 254 nm.

Draize Method

The hair-shaved guinea pig was anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), and a cotton pad with a diameter of 11.0 mm and a thickness of 0.75 mm containing 100 μL of each concentration (0, 1, 2, 5, 7.5, 10, and 20%) of CPC in physiological saline solution was applied on four sites of the abdominal skin. A physiological saline (0% CPC) sample was applied at one site as a control. Adhesive tape was used to cover the cotton pad so as not to separate it from the animal. Furthermore, the whole abdomen was covered with a bandage to ensure the application of the CPC solution. After 48 h, the cotton pad was removed from the abdomen and the degree of skin irritation was determined judged for redness and swelling according to the general Draize method (the maximum mark for redness and swelling is 4 points). The skin sample was then excised and used for the MTT assay immediately after the Draize test.

MTT Assay

The extent of skin irritation of the LSE-high and guinea pig skin was evaluated by the calorimetric MTT conversion assay (24). The metabolic reduction of this soluble tetrazolium salt to a blue formazan precipitate is dependent on the presence of viable cells with intact mitochondorial function (25). The MTT assays performed in this study essentially followed the procedure defined for monolayer cell cultures (26), modified to suit the LSE-high and guinea pig skin. Each concentration of CPC was applied to the stratum corneum side of LSE-high for 24 h at 37°C in a humidified atmosphere (95% O_2 and 5% CO_2). The CPC concentrations tested were 1–20% for guinea pig skin and 0.01–1.0% for LSE-high. The CPC solution on the LSE-high or guinea pig skin was removed by a pipette and washed with 1.0 mL of the assay medium (Tsuruga Institute of Biotechnology, Toyobo Co., Ltd.) for LSE-high. The 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 skin. After reaction for 3 h at 37°C in a humidified atmosphere, the tissue sample was washed with 1.0 mL of the assay medium. A tissue disc having a diameter of 8.0 mm was made by biopsy punch. The obtained tissue was transferred to a test tube and was submerged in 1.0 mL of 0.04 N HCl in isopropanol. The formazan produced from the MTT conversion was extracted overnight at room temperature in the dark. The absorbance of the solution was measured at 570 nm using a spectrophotometer (UV-160A, Shimadzu).

RESULTS

Figure 1(a) and (b) show the time course of the cumulative amount of CPC that permeated through the LSE-high and guinea pig skin, respectively. The CPC concentration applied was 1% on the LSE-high and 5% on the guinea pig skin. The *in vitro* membrane permeation study was done using full-thickness (intact) skin as well as stripped skin to evaluate the variability in the barrier function of the membranes tested. Stripped skin was used as a typical low barrier model. As

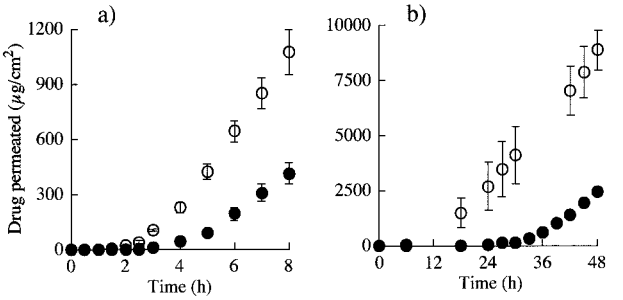

Fig. 1. Time course of changes in the cumulative amount of CPC that permeated through LSE-high (a) and guinea pig skin (b). Symbols in the figure are as follows: (●) full-thickness skin; (○) stripped skin. The LSE-high or excised guinea pig skin was mounted in a two-chamber diffusion cell. CPC solution in physiological saline was applied to the epidermal side, and physiological saline was applied to the dermal side. At predetermined time points, aliquots were sampled from the dermal side to measure the CPC permeation. Each point represents the mean ± SE of 3 experiments.

Table I. Permeability Coefficient of Cetylpyridinium Chloride in Each Skin Layer and Permeation Parameter (RR^a and K_{ved}^b)

	LSE-high	Guinea pig
P_{tot} ($\times 10^{-6}$cm/s)	2.569	0.8891
P_{sc} ($\times 10^{-6}$cm/s)	4.58	2.249
P_{ved} ($\times 10^{-6}$cm/s)	5.851	1.471
RR	0.439	0.604
K_{ved}	11.291	5.487

[a] RR was calculated from Eq. 3.

[b] K_{ved} was obtained by curve fitting of the permeation data through the skin to Eq. 4, then L_{ved} was set to 530 μm for LSE-high and 1130 μm for guinea pig.

expected, the stripped skin permeation (○) showed a shorter lag time and a higher following steady state flux than the intact skin permeation (●) both in the LSE-high and guinea pig skin. Under the assumption that both LSE-high and guinea pig skin consist of two independent single diffusion membranes, stratum corneum and viable epidermis and dermis, the permeability coefficient in the viable epidermis and dermis, P_{ved}, was determined using Eq. (4) as $K_{ved} \cdot D_{ved}/L_{ved}$. P_{sc} was then determined by the permeation profiles across the full-thickness skin and stripped skin, and RR and K_{ved} were determined using Eqs. (2) and (4), respectively. The results obtained are summarized in Table I. These parameters (P, D, RR, and K_{ved}) were supposed to be independent of CPC concentration applied on skin.

The permeability coefficients of CPC, P_{tot}, P_{sc}, and P_{ved} for LSE-high were 2.8-, 2.0-, and 4.0-fold higher, respectively, than those for the guinea pig skin (P_{tot} is permeability coefficient of CPC through full-thickness skin). RR for LSE-high, 0.439, was much higher than zero like that for the guinea pig skin, 0.604, suggesting that permeation of CPC through these skins was greatly affected by the barrier function of the viable epidermis and dermis as well as that of the stratum corneum. K_{ved} for LSE-high was about twice as that in the guinea pig skin.

Figure 2 shows the *in vivo* irritation scores (Draize scores) against the CPC concentration applied [(a) and (b)] and that at the irritation site (c). As explained in the theoretical section, Draize score is expressed in a magnitude scale in general. We treated it as a continuous quality according to previous works (17,18). Full-thickness skin as well as stripped skin was used to evaluate the effect of variability in the skin barrier function among the test membranes on the skin irritation. As expected, the irritation score became higher with an increase in the irritant concentration to reach maximum irritation. When compared with the same applied concentration, the average Draize score in the stripped skin [Fig. 2(a)] was much higher than that in the full-thickness skin [Fig. 2(b)]. When the Draize scores were plotted against the CPC concentration at the irritation site, however, the data using stripped skin could be expressed in the same figure [Fig. 2(c)] as in the data using full-thickness skin. The solid line in Fig. 2(c) is the theoretical curve [fitting line to the Hill equation, Eq. (5)]. Thus, these *in vivo* irritation scores were well represented as a function of CPC concentration at the irritation site according to the toxicokinetics-toxicodynamics relation. It is suggested from the results that application of CPC concentration at the reaction site was very useful to evaluate the Draize score without considering the variability of barrier function tested. Table II summarizes the obtained parameters.

Figure 3 shows the *in vitro* irritation scores (% fraction of dead cell number in the viable epidermis and dermis obtained by the MTT assay) against the CPC concentration applied [(a) and (b)] and that at the irritation site (c). The solid line in Fig. 3(c) shows the fitting curve to the Hill equation. The obtained parameters for the *in vitro* skin irritation are also shown in Table II. All the *in vitro* irritation scores were also well represented as a function of the CPC concentration at the irritation site. The obtained IC_{50} for the *in vivo* and *in vitro* irritation measured by the Draize test and MTT assay in the guinea pig skin showed the same order of magnitude (12.0 and 18.9%, respectively).

Figure 4 shows the relationship between the results for the Draize test and MTT assay in guinea pigs. A very good correlation was obtained ($r = 0.981$). As expected, a higher *in vitro* skin irritation score (% of dead cell number in the viable skin) showed a higher *in vivo* irritation score (Draize score). The relation was as follows:

$$\text{Draize score} = 2.820 \times 10^{-2} \cdot \%\ \text{fraction of dead cell number} + 1.272 \quad (6)$$

Figure 4 and Eq. (6) suggest that the *in vitro* irritation test using the MTT assay can be utilized to predict the *in vivo* Draize irritation in guinea pigs.

Figure 5(a,b) show percent fraction of dead cell number determined by the MTT assay using stripped and intact LSE-high, respectively, against the CPC concentration applied. Figure 5(c) shows the same data but against the CPC concentration at the irritation site. The solid line in the figure shows

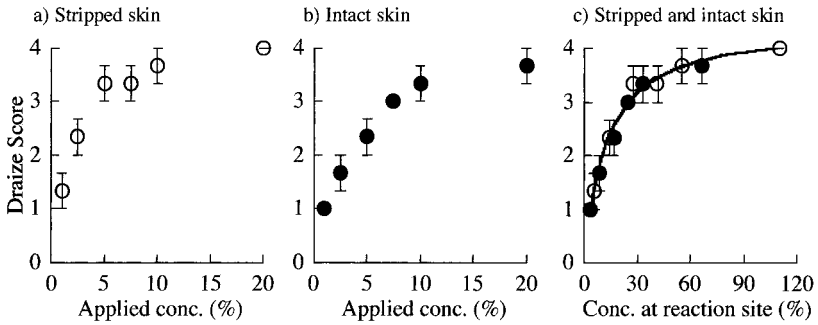

Fig. 2. Dose-response curve for the Draize score and CPC concentration applied (a,b) and at the reaction site (c) in guinea pig skin. Fig. 2a and b show the results for the stripped skin and full-thickness skin, respectively. Symbols in the figure are as follows: (●) intact skin; (○) stripped skin; solid lines, fitting line to Hill equation.

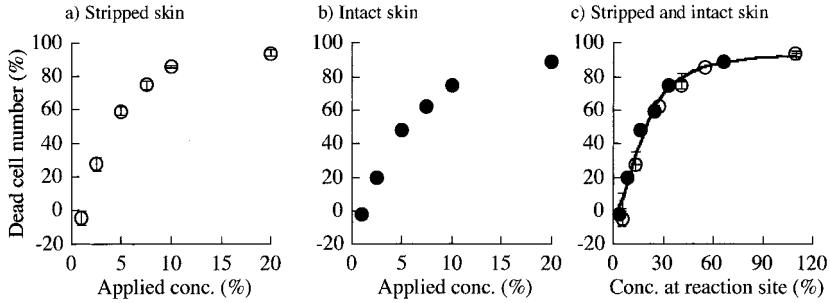

Fig. 3. Dose-response curve for percent of dead cell number in the MTT assay and CPC concentration applied (a,b) and at the reaction site (c) in guinea pig skin. Fig. 3a and b show results for the stripped skin and full-thickness skin, respectively. Symbols in the figure are as follows: (●) intact skin; (○) stripped skin; solid lines, fitting line to Hill equation.

the theoretical value to the Hill equation. LSE-high can be used as well as the guinea pig skin for evaluating the skin irritation. Application of *RR* was useful to evaluate the data using full-thickness skin at the same time as the data using stripped skin. Because the MTT conversion data in the LSE-high showed a similar profile to that in the guinea pig skin, the MTT assay using LSE-high can be utilized for the Draize test in guinea pigs like for the MTT test in guinea pig skin.

DISCUSSION

Although skin irritation has been reported to be dependent on the irritant amount applied on the skin, the irritation must be more directly dependent on the irritant concentration at the reaction site of irritation. No report on the relationship between the extent of skin irritation and the irritant concentration at the reaction, however, was found. This may be due to the difficulty in measuring the irritant concentration in the skin. In the present study, we estimated the irritant concentration in skin from its *in vitro* skin permeation profile. The skin concentration of CPC must increase with time after application to become a steady-state level, as predicted by the results in Fig. 1. Skin irritation was evaluated at 24 and 48 h for LSE-high and guinea pig skin, respectively. CPC concentration in skin at these times must be a steady-state level. The obtained relationship between the *in vivo* or *in vitro* irritation index (Draize score or fraction of dead cell number in the skin membrane, respectively) and the calculated irritant concentration in the skin at steady state was evaluated by the Hill equation, Eq. (5). The present strategy is closely related to the view of toxicokinetics and toxicodynamics. Skin irritation may be related to the cumulative amount of irritant exposed on the viable skin tissues, i.e., skin irritation increases with time even if the irritant concentration in the viable skin is constant. We then studied the time course of skin irritation and reported them in a separate paper (27).

Significant relationships represented by the Hill equation were observed between the skin irritation scores and the concentration at the irritation site of CPC, as shown in Figs. 2, 3, and 5. These findings suggest that skin irritation is a biologic reaction represented by a general dose-response curve.

When the Draize score or the fraction of the dead cell number in the MTT assay was plotted against the applied concentration of CPC, the obtained relationships were different between the data for the full-thickness skin [Figs. 2(b), 3(b), and 5(b)] and the stripped skin [Figs. 2(a), 3(a), and 5(a)] on which the calculated CPC solution was applied. When these irritation scores were plotted against the CPC concentration in viable skin, however, both data for the full-thickness skin and the stripped skin could be plotted in the same figure and expressed by one Hill equation [Figs. 2(c), 3(c), and 5(c)]. This similarity was obtained by applying the permeation resistant ratio of the viable epidermis and dermis against that of the full-thickness skin, *RR*. Skin irritation in

Table II. Skin Irritation Parameters for Cetylpyridinium Chloride on MTT Assay and Draize Score

	LSE-high	Guinea pig
(a) MTT		
I_{max} (%)	93.464	94.148
IC_{50} (%)	1.315	18.868
γ	1.411	2.138
(b) Draize		
I_{max}	—	4.466
IC_{50} (%)	—	12.312
γ	—	1.043

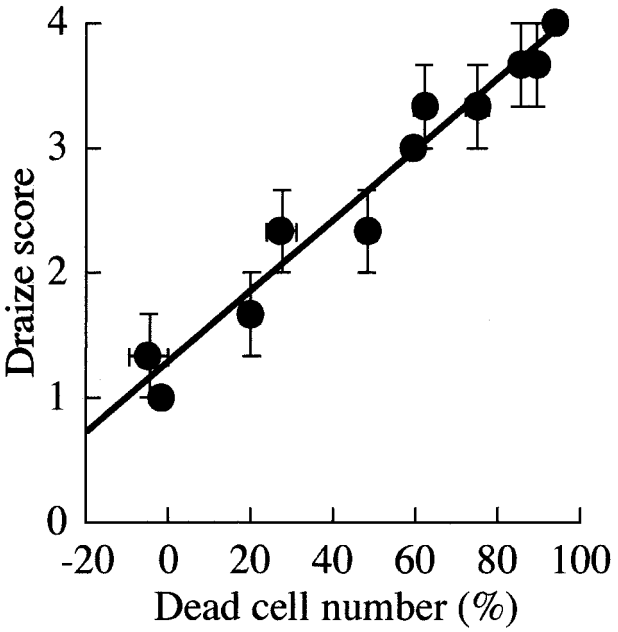

Fig. 4. Relationships between the Draize score and percent of dead cell number in the MTT assay using guinea pig skin. Solid lines represent the estimated curve, Draize score = $2.820 \times 10^{-2} \cdot$ % fraction of dead cell number + 1.272.

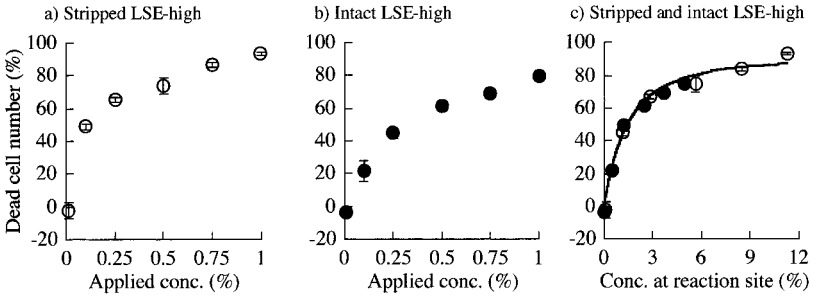

Fig. 5. Dose-response curve for percent of dead cell number in the MTT assay and CPC concentration applied (a,b) and at the reaction site (c) in LSE-high. (a) and (b) show results for the stripped skin and full-thickness skin, respectively. Symbols in the figure are as follows: (●) intact skin; (○), stripped skin; solid lines, fitting line to Hill equation.

patients with a topic dermatitis may be predicted by that in healthy volunteers using permeation resistances of the irritants in both the skin barriers. Further experiment must be done to see a relationship between the skin irritation and actually determined concentration of an irritant in the viable skin.

A good relationship was observed between the Draize score and percent of dead cell number in the viable skin measured by the MTT assay in guinea pigs (Fig. 4). This is because IC_{50} in the Draize test and the MTT assay in the guinea pig were similar to each other (12.3% and 18.9%, respectively). These findings suggest that the MTT assay is an alternative for the Draize test.

In contrast, the comparison of the MTT assay in guinea pig skin and LSE-high showed that IC_{50} was different despite similar I_{max} and γ. This may be due to a higher permeability through the cell membrane in LSE-high than that in guinea pig skin. The higher the cell membrane permeability, the greater is the obtained skin irritation. The higher permeability of CPC across the cell membrane in LSE-high than in guinea pig skin may be related to the different irritation scores by the MTT assay in both skin samples. Thus, the CPC concentration in the viable tissue of LSE-high was corrected by IC_{50} in the guinea pig / IC_{50} in LSE-high, as shown in Fig. 6. The obtained figure shows similar profiles for both the irritation scores by the MTT assay in the guinea pig skin and LSE-high. Fig. 6.

The CPC concentration at the reaction site was much higher in the Draize test (Fig. 2) and MTT assay (Fig. 3) in guinea pigs than that in the MTT assay using LSE-high (Fig. 5). The highest CPC concentration at the reaction site in guinea pigs was over 100%, which is nonrealistic. This is because the present concentration in the reaction site was obtained just by calculation under the assumption that the viable epidermis and dermis is a homogenous single membrane. In general, the diffusivity of most drugs and materials in the epidermis is much lower than that in the dermis. Therefore, the present CPC concentration must have been overestimated. These values, however, are used as an index. The present objective of this study was not affected by this problem. Direct determination of the irritant concentration in the viable skin may solve this problem.

In the present study, CPC was used as a model irritant due to easy determination by HPLC. CPC is a cationic surfactant in which the critical micelle concentration (c.m.c.) is 0.29 mg/ml. The c.m.c value was much lower than the CPC concentration applied on the skin (1–20%). No information on the effect of the c.m.c. value on the skin irritation was obtained in the present study.

The rate limiting step in the skin permeation of most therapeutic drugs is in the diffusion across the stratum corneum, whereas that of CPC is diffusion in the stratum corneum as well as that in the viable epidermis and dermis. Thus, CPC may have a penetration-enhancing effect on the permeation through the stratum corneum as most surfactants have.

Skin permeation rate and toxicokinetics of irritants are also important issues to be determined. These must be closely related to the present objectives. The kinetic approach is shown in our separated paper (27).

The comparison of different irritants is an important topic. Preliminary studies on the MTT assay in LSE-high were done using several surfactants. No significant differences were observed in the I_{max} and γ values among the surfactants. IC_{50} may be an important parameter. Further

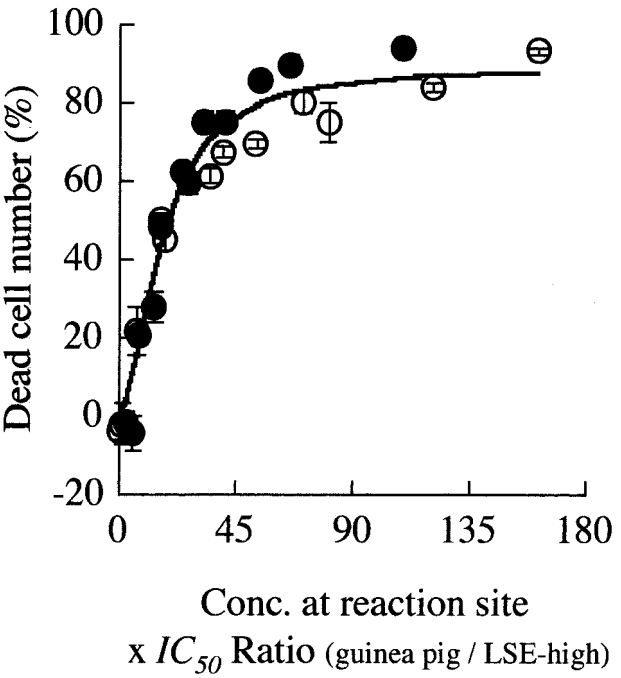

Fig. 6. Relationship between irritation scores from the MTT assay in guinea pig and LSE-high. Symbols in the figure are as follows: (●), Guinea pig skin; (○), LSE-high; solid lines, fitting line CPC concentration in the viable tissue of LSE-high was corrected by IC_{50} in guinea pig / IC_{50} in LSE-high.

experiments should be done using different kinds of irritants and various dosage forms. The present study, however, suggests that the MTT assay using LSE-high can be used as an alternative for *in vivo* skin irritation tests such as the Draize test in rabbits and guinea pigs. The present method is a useful tool to evaluate skin irritation by pharmaceutical and cosmetic materials.

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