Evaluation of Skin Damage of Cyclic Monoterpenes, Percutaneous Absorption Enhancers, by Using Cultured Human Skin Cells

Megumi KITAHARA,[a] Fumiko ISHIGURO,[b] Kozo TAKAYAMA,*[,a] Koichi ISOWA,[c] and Tsuneji NAGAI[a]

Department of Pharmaceutics, Hoshi University,[a] Ebara-2-4-41, Sinagawa-ku, Tokyo 142, Japan, Taikyo Pharmaceutical Co., Ltd.,[b] 13-13, Mizuhashi-Showa-cho, Toyama 939-05, Japan and The Center of Japan Biological Chemistry Co., Ltd.,[c] Fukue Kaizu-cho, Kaizu-gun, Gifu 503-06, Japan. Received March 2, 1993

The cytotoxicity of monoterpenes, percutaneous absorption enhancers, to cultured human skin cells was investigated in order to quantitatively estimate their skin damage. A neutral red bioassay with epidermal keratinocytes and a contraction test of collagen gel in which dermal fibroblasts were cultured were employed for evaluating the cytotoxicity of terpenes. In the neutral red bioassay, keratinocyte proliferation was inhibited on the addition of terpenes, and cell survival remarkably decreased with an increase in the concentration of terpenes fed into the culture well. When the fibroblasts were cultured in a collagen gel matrix, the lattice of collagen contracted as the cells grew. Therefore, the application of cytotoxic agents brings about an inhibition of collagen gel contraction induced by the fibroblasts. Strong inhibition was observed in the cases of hydrocarbons in terpenes, and the inhibition was dependent on the concentration of these compounds added in the culture medium. The cytotoxicity of terpenes was compared with the skin damage evoked by the application of terpenes in rats *in vivo*. As a result, it was considered that the skin irritation caused by terpenes was predictable to a certain extent by means of the cytotoxic study of cultured human skin cells.

Keywords keratinocyte; fibroblast; cytotoxicity; cyclic monoterpene; skin damage

A transdermal drug delivery system, which can provide an effective means of drug administration, may be developed by promoting the drug permeation across the skin in combination with certain materials. Cyclic monoterpenes present in essential oils have been reported to enhance drug permeation through the skin under the coexistence of ethanol.[1-3] However, the skin irritancy of these compounds should be investigated in detail, in addition to their enhancing activity. The skin irritation of chemicals has generally been evaluated by animal experiments. The use of animals in toxicity tests is costly, time-consuming, and labor intensive. Recently, new culture techniques involving human cells have received attention as an alternative to animal testing, and they are expected to reduce the number of animals needed.[4-7] Ponec *et al.* employed cultured human skin cells in order to estimate the skin toxicity of *n*-alkylazacycloheptan-2-one and other compounds.[8,9] In this study, we investigated the cytotoxicity of cyclic monoterpenes on cultured human keratinocytes and fibroblasts, and attempted to predict skin irritation brought about by the application of these penetration enhancers.

Materials and Methods

Materials A secondary culture of normal human epidermal keratinocytes (designated NHEK and purchased from Kurashiki Bouseki Co., Ltd.) was maintained in a serum-free, biochemically defined keratinocyte growth medium (KGM), which is a modified MCDB 113 formulation supplemented with 10 ng/ml of epidermal growth factor, 5 μg/ml of insulin, 0.5 μg/ml of hydrocortisone, 0.15 mM Ca^{2+}, 0.4 (v/v)% bovine pituitary extract (BPE) and antibiotics. A secondary culture of normal human dermal fibroblasts (designated NHDF and purchased from Kurashiki Bouseki Co., Ltd.), was maintained in a serum-free, biochemically defined fibroblast growth medium (FGM), which is a modified MCDB formulation supplemented with 1 ng/ml of fibroblast growth factor, 5 μg/ml of insulin, and antibiotics. A stock collagen solution (Cellmatrix Type I-A) was purchased from Nitta Gelatin Co., Ltd. The chemical structures of cyclic monoterpenes used in this study are shown in Fig. 1. The compounds were of extra pure reagent grade, and were purchased from Tokyo Chemical Industries Co., Ltd. Carboxyvinyl polymer, marketed as Hiviswako 105, was purchased from Wako Pure Chemical Industries, Ltd.

Neutral Red (NR) Bioassay[7,10] The cytotoxicities of terpenes were determined with a neutral red bioassay. This assay quantifies the number of viable, uninjured cells after their exposure to terpenes; it is based on the uptake and subsequent lysosomal accumulation of the supravital dye, NR. Quantitation of dye extracted from the cells has been shown to be linear with cell numbers, both by direct cell counts and by protein determination of the cell populations. Cell survival was defined as quantity of NR uptaken by viable cells (% of control). Individual wells of a 96-well tissue culture microtiter plate were inoculated with 250 μl of the appropriate KGM containing cells and then incubated for 3 d. The medium was then removed and the cells were refed with unamended medium (control) or with a medium amended with varied concentrations of terpenes. After 2 d of exposure, the medium was removed and replaced with 250 μl of medium containing 50 μg/ml of NR. The medium containing neutral red had been preincubated at 37 °C and centrifuged prior to use in order to remove fine precipitates of dye crystals. The plate was returned to the incubator for another 3 h to allow for the uptake of the vital dye into the lysosomes of viable, uninjured cells. Thereafter, the medium was removed and the cells were washed quickly with a fixative (1% $CaCl_2$:

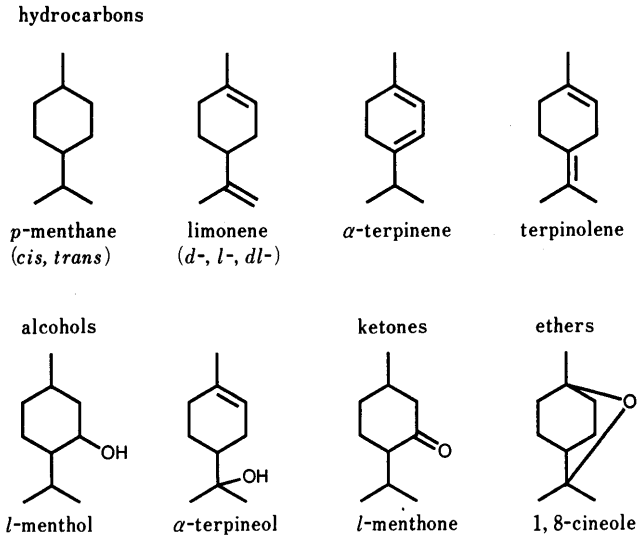

Fig. 1. Chemical Structures of Cyclic Monoterpenes Used in This Study

1% formaldehyde), and then 100 μl of a solution of 1% acetic acid: 50% ethanol was added to each well to extract the dye. After an additional 20 min and rapid agitation, the concentration of the extracted dye was determined by a microtiter plate reader (Model 450, Biorad Laboratories) at 540 nm.

Collagen Contraction Assay[9,11] Fibroblasts were harvested by trypsinization and the cell number was determined. On ice, a mixture was made of 0.744 ml of collagen stock solution, 0.2 ml of buffer (50 mM NaOH, 260 mM $NaHCO_3$ and 200 mM HEPES) which produces a neutral mixture, 0.128 ml of Eagle's MEM (concentrated 10 times), 0.2 ml of phosphate buffered saline (PBS) and 0.728 ml of cell suspension in Eagle's MEM. The mixture was poured into a Corning tissue culture dish (diameter 35 mm) and placed in an incubator at 37 °C. The gelation started immediately. After 1 h, the formed gel was loosened by shaking the dishes. The diameter of the collagen gel was measured by placing the dish on a black background bearing a ruler. The measurement was repeated twice. The morphology of the cells was observed by phase-contrast microscopy. An inhibition ratio of collagen gel contration by the addition of cyclic monoterpenes was expressed as the ratio to the control.

Evaluation of Skin Irritation in Rats The formulae of the gel ointments used in this study are listed in Table I. The gel ointments were prepared as follows: Cyclic monoterpenes were dissolved in ethanol. Separately, carboxyvinyl polymer and triethanolamine were dissolved in distilled water. Both compounts were then mixed well and the resulting gel ointment was stored at room temperature for 24 h under air-tight conditions prior to use.

Male Wistar rats weighing 160—190 g were used. After anesthetization with a urethane saline solution (25%, 3 ml/kg i.p.), the rats were secured on their backs and the hair on the abdominal skin was removed with an electric animal clipper. Glass cells (16 mm inner diameter, 10 mm height) containing the gel ointment under test (1.5 g) were attached to the shaved skin with cyanoacrylate-type adhesives. After 10 h, the gel ointments were removed. Two of the dosing sites on the skin for each preparation were immediately taken from the rats (each sample was about 1 cm^2 of area). The excised skin was fixed in 10% neutral carbonate-buffered formalin for at least 24 h before routine procession and then cut vertically against the skin surface at the central region in 4 mm widths. Each section was

TABLE I. Formulae of Gel Ointments Containing Cyclic Monoterpenes

Carboxyvinyl polymer	2.0 g
Triethanolamine	2.5 g
Ethanol	50.0 g
Cyclic monoterpenes	2.0 g
Water	ad. 100.0 g

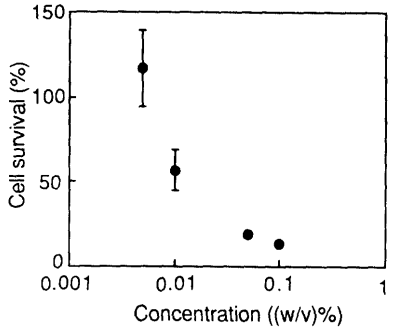

Fig. 2. Effect of Concentration of *d*-Limonene on Survival of Human Keratinocytes Cultured for 2 d in the Presence of 0.5 (v/v)% Ethanol

Each point represents the mean ± S.E. of four determinations.

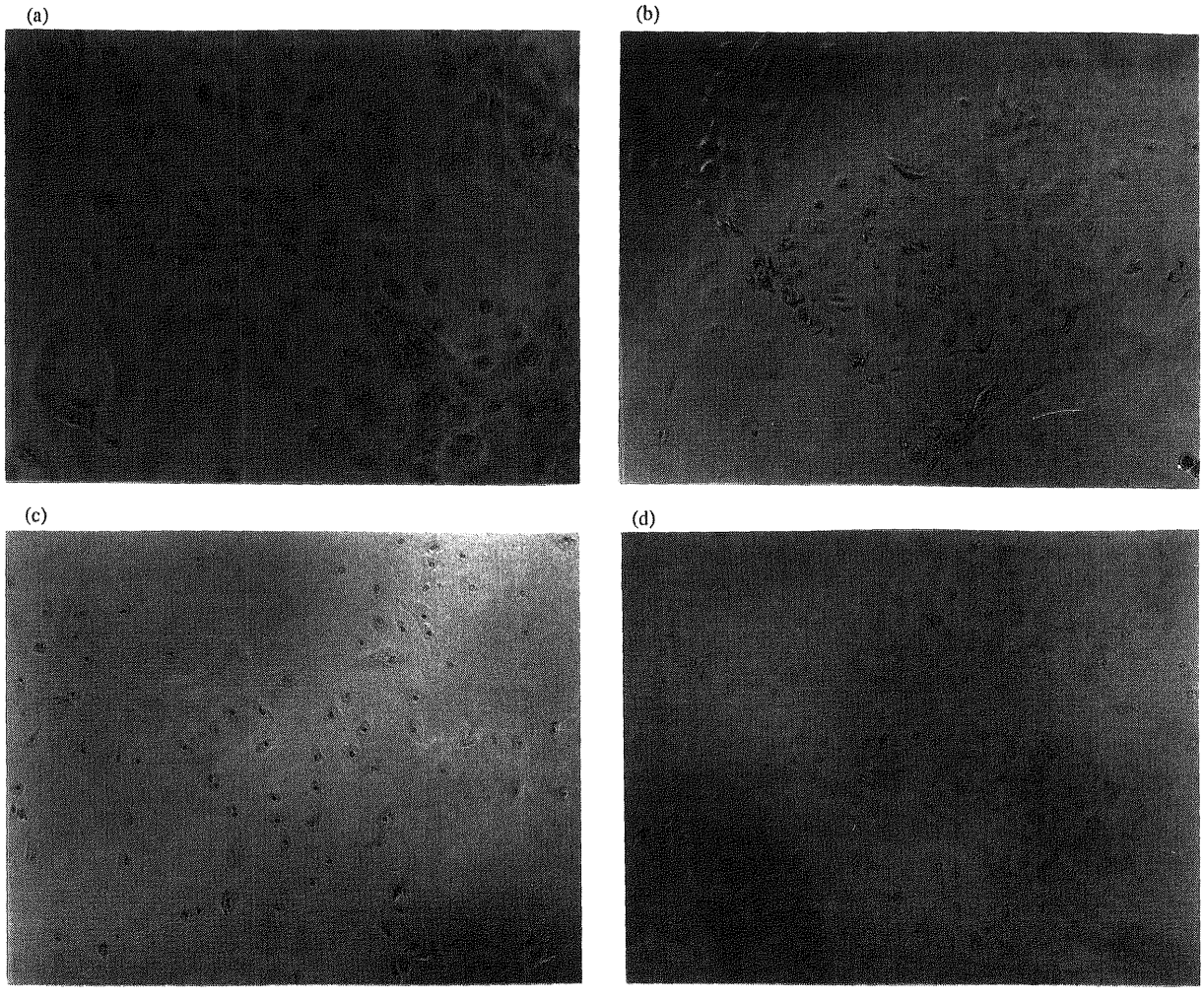

Fig. 3. Microscopic Photos of Human Keratinocytes Cultured for 2 d in the Presence of 0.005—0.1 (w/v)% *d*-Limonene and 0.5 (v/v)% Ethanol (× 100)

Concentration of *d*-limonene: (a) 0.005 (w/v)%; (b) 0.01 (w/v)%; (c) 0.05 (w/v)%; (d) 0.1 (w/v)%.

dehydrated using a graded series of ethanol solutions, and was then embedded in paraffin wax. Tissues were divided into small pieces (about 3 μm in thickness) and stained with hematoxylin and eosin. All sections were examined by an optiphoto light microscope (Optiphoto, Nikon).

Results and Discussion

The cell survival of keratinocytes and the inhibition ratio in fibroblasts were determined as quantitative values. Figure 2 exemplifies a typical concentration–response cytotoxicity curve generated by the NR bioassay. Keratinocyte proliferation was inhibited on the addition of *d*-limonene, and cell survival decreased remarkably with an increase in the concentration of terpene fed into the culture well. The change in cell morphology was followed on a light microscopic level (Fig. 3). When NHEK were incubated in the presence of 0.005 (w/v)% *d*-limonene and 0.5 (v/v)% ethanol for 2 d, the cells were extended, attached, and similar in shape to the control cells. However, cellar atrophy was observed when the cells were incubated in the presence of 0.1 (w/v)% *d*-limonene and 0.5 (v/v)% ethanol for 2 d. When NHDF were incorporated into collagen gel lattices, the lattice contracted in accompani-

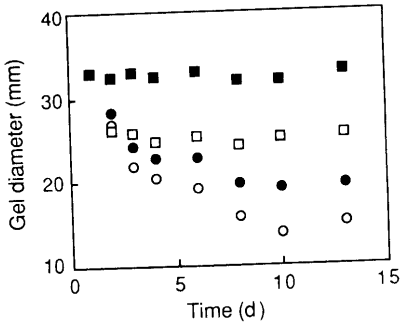

Fig. 4. Collagen Gel Contraction Induced by Human Fibroblasts Cultured in the Presence of 0.1 (w/v)% Terpenes and 0.5 (v/v)% Ethanol as a Function of Time

○, control (untreated); ●, 1,8-cineole; □, *l*-menthol; ■, *d*-limonene. Each value represents the mean of two determinations.

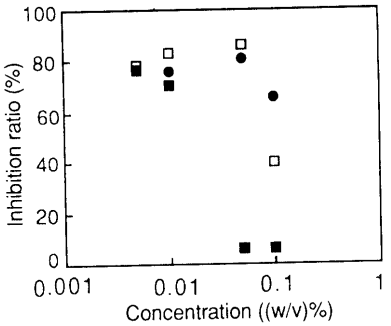

Fig. 5. Effect of Concentration of Terpenes on Collagen Gel Concentration Induced by Human Fibroblasts Cultured for 10 d in the Presence of 0.5 (v/v)% Ethanol

●, 1,3-cineole; □, *l*-menthol; ■, *d*-limonene. Each value represents the mean of two determinations.

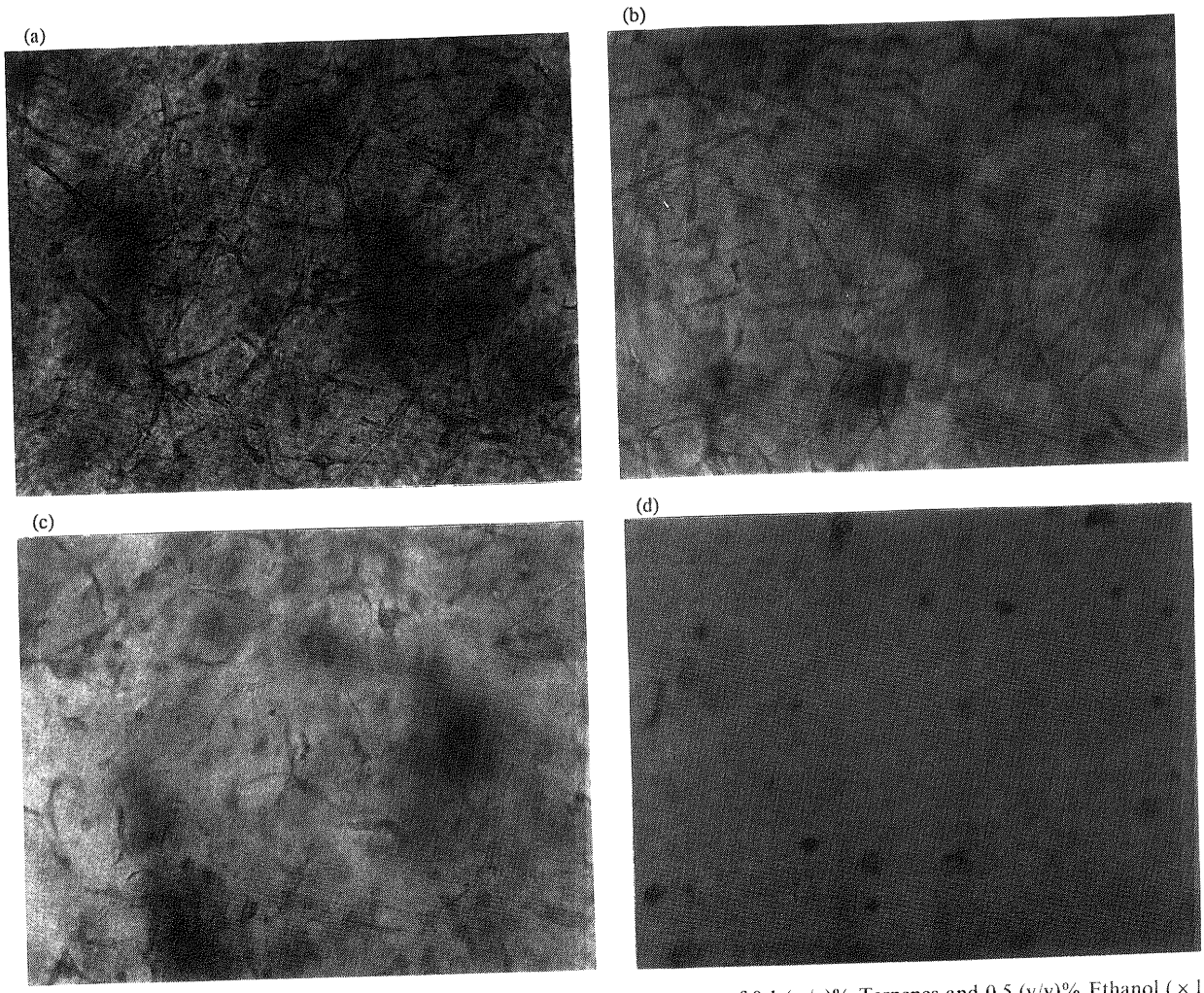

Fig. 6. Microscopic Photos of Human Fibroblasts Cultured for 10 d in the Presence of 0.1 (w/v)% Terpenes and 0.5 (v/v)% Ethanol (× 100)

(a) Control (untreated), (b) 1,8-cineole, (c) *l*-menthol, (d) *d*-limonene.

ment with cell growth (Fig. 4). The extent of lattice contraction was reduced on the addition of terpenes and ethanol. It was observed that the inhibition of collagen gel contraction was dependent on the concentration of terpenes (Fig. 5). The morphology of cells treated with terpenes was distinguishable from that of the control (Fig. 6). In the control gel, cells extended well, whereas many round cells were observed in the terpene-treated gel.

In order to compare the cytotoxicity of terpenes *in vitro* with the skin damage *in vivo*, gel ointments containing terpenes were applied to the rat abdominal skin. Histopathological findings and irritation scores are summarized in Table II. For example, microscopic photos of rat skin at 10 h after application of gel ointments are shown in Fig. 7. The three layers of skin tissue (epidermis, dermis and hypodermis) showed no significant change in the case of the control ointment without terpenes (Fig. 7a). Rat skin treated with α-terpineol showed very slight damage: collagen fiber swelling in the stratum papillare of the dermis (Fig. 7b). When gel ointments containing hydrocarbon terpenes were applied to the rat skin, these compounds showed relatively strong skin irritation. The rat skin treated with *d*-limonene showed epidermal liquefaction, desquamation, collagen fiber swelling in the stratum papillare and reticulare of the dermis and hypodermis with hypodermal edema (Fig. 7c).

As a quantitative measurement of the effect of terpenes, the following indices were taken: Survival of NHEK cultured for 2 d in the presence of 0.05 (w/v)% terpenes and 0.5 (v/v)% ethanol; and the inhibition ratio of collagen gel contraction induced by NHDF cultured for 10 d in the presence of 0.1 (w/v)% terpenes and 0.5 (v/v)% ethanol. These indices are summarized in Table III. As a result, the survival of cultured NHEK were inversely proportional to

TABLE II. Histopathological Findings of Rat Abdominal Skin at 10 h after Application of Gel Ointments Containing Cyclic Monoterpenes

Histopathological findings	Cyclic monoterpenes[a]											
	Control	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Epidermis												
Liquefaction	0	0	0	0	1	3	3	3	3	3	3	4
Desquamation	0	0	0	0	0	3	3	3	3	3	3	3
Dermis												
Collagen fiber swelling												
in stratum papillare	0	0	0	1	2	2	3	3	2	2	3	3
in stratum reticulare	0	0	0	0	1	2	3	3	2	3	3	2
Hypodermis												
Collagen fiber swelling	0	0	0	0	2	3	4	4	4	3	4	3
Focal hemorrhage	0	0	0	0	0	1	0	1	1	0	1	0
Edema	0	0	0	0	0	3	3	3	3	3	3	3
Skin appendages												
Degeneration	0	0	0	0	1	4	4	4	4	4	4	3

a) Cyclic monoterpenes: I, 1,8-cineole; II, *l*-menthol; III, α-terpineol; IV, *l*-menthone; V, *trans-p*-menthane; VI, terpinolene; VII, *dl*-limonene; VIII, α-terpinene; IX, *l*-limonene; X, *d*-limonene; XI, *cis-p*-menthane. Irritation score: 0, no change; 1, very slight; 2, slight; 3, moderate, 4, marked. Each datum represents the average value of three to four determinations.

TABLE III. Effect of Cyclic Monoterpenes on the Cell Survival of NHEK and the Inhibition Ratio of the Collagen Gel Contraction Induced by NHDF

Cyclic monoterpene	Survival of NHEK[a] (%)	Inhibition ratio of NHDF[b] (%)
1,8-Cineole	105.1	20.3
l-Menthol	38.9	51.3
α-Terpineol	83.5	74.3
l-Menthone	76.4	65.7
trans-p-Menthane	5.64	97.8
Terpinolene	26.9	52.4
dl-Limonene	27.4	65.7
α-Terpinene	39.2	77.0
l-Limonene	26.6	88.4
d-Limonene	17.7	92.8
cis-p-Menthane	9.11	97.8

a) Each datum represents the cell survival of NHEK cultured for 2 d in the presence of 0.05 (w/v)% terpenes and 0.5 (v/v)% ethanol (mean ± S.D. of four determinations). *b*) Each datum represents the inhibition ratio of collagen gel contraction induced by NHDF cultured for 10 d in the presence of 0.1 (w/v)% terpenes and 0.5 (v/v)% ethanol (mean of two determinations).

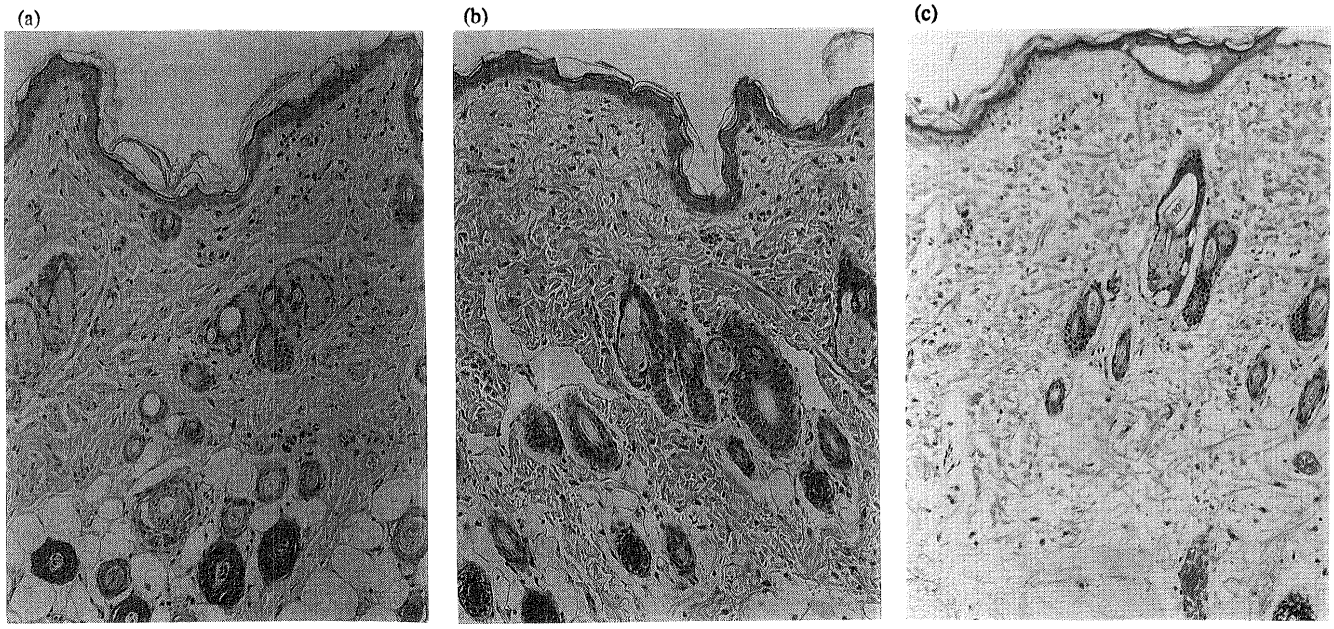

Fig. 7. Microscopic Photos of Rat Skin at 10 h after Application of Gel Ointments Containing 2 (w/v)% Terpenes and 50 (w/v)% Ethanol (H and E Stain, × 100)

a) Control (without enhancer), (b) α-terpineol, (c) *d*-limonene.

the sum of irritation scores in epidermis liquefaction and desquamation ($r=-0.85$). Whereas, the inhibition ratio of the collagen gel contraction induced by the cultured NHDF was positively correlated to the total value of irritation scores in the dermis, though the correlation coefficient was not sufficient ($r=0.57$). These results may suggest that the skin irritation evoked by terpenes can be evaluated to a certain extent by using cultured human skin cells.

According to the irritation scores summarized in Table II, the irritating action of terpenes to the whole skin was roughly classified into two categories. Namely, class 1 is the group showing no change or very slight irritation, and class 2 is the group exhibiting definite irritation. Relatively hydrophilic terpenes such as alcohols, ethers and ketones were classified in the low irritation group (class 1). On the other hand, hydrocarbon terpenes were classified as class 2 since these compounds exhibited definite irritation under these experimental conditions. In order to predict skin irritation using the results obtained with the cell culture experiments, the adaptive least-squares (ALS) method[12] was empolyed. The ALS method was developed in the field of research dealing with quantitative structure–activity relationships to the help make decisions regarding multicategory pattern classification by a single discriminant function in those cases where activity data are given as an ordinal scale. The discriminant function for the skin irritancy (L), $L=-0.00991D_1+0.00656D_2+1.01$ was obtained. D_1 represents the cell survival of NHEK, and D_2 is the inhibition ratio of the collagen gel contraction induced by NHDF. The irritation score of each terpene was predicted in the following manner: If $L_n \leq 1$ (n: number of terpenes), the n-th terpene was assigned to class 1; for $L_n > 1$, the n-th terpene was assigned to class 2. As a result, classification based on discriminant functions was observed to be perfect. This fact may suggest that the cytotoxic assay of cultured human skin cells is quite useful for evaluating skin damage evoked by percutaneous absorption enhancers such as cyclic monoterpenes.

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