Altern. Animal Test. Experiment. 6 (1),41-51 (1999)

Alternative to Primary Draize Skin Irritation Test Using Cultured Human Skin Model: Comparison of Six Endpoints

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Abstract

In this study, we evaluated skin irritancy of test chemicals by using six endpoints. As an endpoint, the cell viability was evaluated with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and neutral red (NR). In addition, lactate dehydrogenase (LDH), interleukin-1α (IL-1α), interleukin-8 (IL-8), and prostaglandin E_2 (PGE_2) releases from the cells were measured. Each assay was compared with *in vivo* animal testing and with one another. The results indicated the assays could be divided into 3 groups, MTT/NR, LDH/IL-1α, and IL-8/PGE_2. The MTT and NR assays revealed good correlation with *in vivo* scores, except for croton oil; the IL-8 and PGE_2 assays could detect the irritancy of croton oil. On the whole, the LDH, IL-1α, IL-8, and PGE_2 assays had poor correlation with *in vivo* scores.

Furthermore, time transition of skin irritancy was examined by the MTT, IL-8, and PGE_2 assays. Production of IL-8 and PGE_2 was related to cell viability as determined by the MTT assay.

This model may be reliable for predicting skin irritancy of chemicals and for studying mechanisms of irritancy by using a combination of endpoints.

Keywords: *skin model, skin irritancy, MTT, NR, LDH, IL-1α, IL-8, PGE_2*

Introduction

During the last decade, an alternative to animal testing using a cultured human skin model has been attempted (Bell et al., 1991, Kuroyanagi et al., 1996, Triglia et al., 1991). Some skin models can be purchased commercially, and these can be used to evaluate skin irritancy caused by chemical reagents, cosmetics, medicines, etc. We have developed a new

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skin model and applied it to evaluations of skin irritancy. This skin model is composed of two types of normal human cells, fibroblasts and keratinocytes, which are cultured on a collagen sponge (Morota et al., 1998).

When such a skin model is used to evaluate skin irritancy, it is important that appropriate endpoints are utilized. Many kinds of endpoint have been proposed, each one having advantages and disadvantages. Some researchers have stated that skin irritancy can be best evaluated by using a combination of various endpoints (Ohno et al., 1995). But which combination is best? Triglia et al. compared 4 endpoints on their dermal model, cell viability determined with neutral red (NR) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and the release of prostaglandin E_2 (PGE_2) and lactate dehydrogenase (LDH) (Triglia et al., 1991). They tested 13 chemicals, but there were no significant differences among the results of the 4 endpoints. On the other hand, Shibata et al. suggested that measurement of interleukin-8 (IL-8) release might be useful to predict the irritancy of chemicals not displaying activity in cytotoxicity assays. These workers showed that IL-8 release was increased with polyethylene glycol fatty acid ester and alkylamidobetaine which would not have been picked up by keratinocyte cytotoxicity testing. However some cytotoxic chemicals, for example, dodecyltrimethylammonium chloride, did not induce IL-8 release (Shibata et al., 1995). This indicates that measurement of IL-8 release alone is not enough to predict skin irritancy.

In this study, we used several test chemicals and evaluated their skin irritancy by employing six endpoints, being cell viability evaluated with MTT and NR, and the release of LDH, interleukin-1α (IL-1α), IL-8, and PGE_2. Each result was compared with *in vivo* animal testing and with each other and result and discussion focuses on combination of endpoints for predicting skin irritancy.

Materials and Methods

Materials

MTT was purchased from Dojindo Laboratories (Kumamoto, Japan) and NR was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Test chemicals were purchased from the indicated sources: Benzethonium chloride (BTC), stearyl trimethyl ammonium chloride (STAC), and olive oil from Nacalai Tesque, Inc. (Kyoto, Japan); sodium lauryl sulfate (SLS) from Wako Pure Chemical Industries, Ltd.; potassium laurate (PL) from Kanto Chemical Co., Inc. (Tokyo, Japan); polyoxyethylene sorbitan monolaurate (TW20), polyoxyethylene sorbitan monooleate (TW80), tributyltin chloride (TBC), and 2, 4-dinitrochlorobenzene (DNCB) from Sigma Chemical Co. (St. Louis, MO, USA); croton oil from Katayama Chemical (Osaka, Japan). All chemicals were used as received.

Dulbecco's phosphate-buffered saline (PBS) was obtained from Wako Pure Chemical Industries, Ltd., and Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Grand Island, NY, USA). K110 medium was obtained from Kyokuto Pharmaceutical Industrial Co., Ltd. (Tokyo, Japan). Human IL-1α ELISA Kit and Human IL-8 ELISA Kit were purchased from Endogen, Inc. (Cambridge, MA, USA), while LDH-Cytotoxic Test Wako and PGE_2 Enzyme Immunoassay Kit were obtained from Wako Pure Chemical Industries, Ltd. and Cayman Chemical Co. (Ann Arbor, MI, USA), respectively.

Preparation of skin model

The skin model was prepared as previously described (Morota et al., 1998). This skin model is composed of two types of collagen sponge and two types of human skin cell, fibroblasts and keratinocytes (Clonetics Corp., Walkersville, MD, USA). Collagen sponges were made from type I collagen (Nitta Gelatin

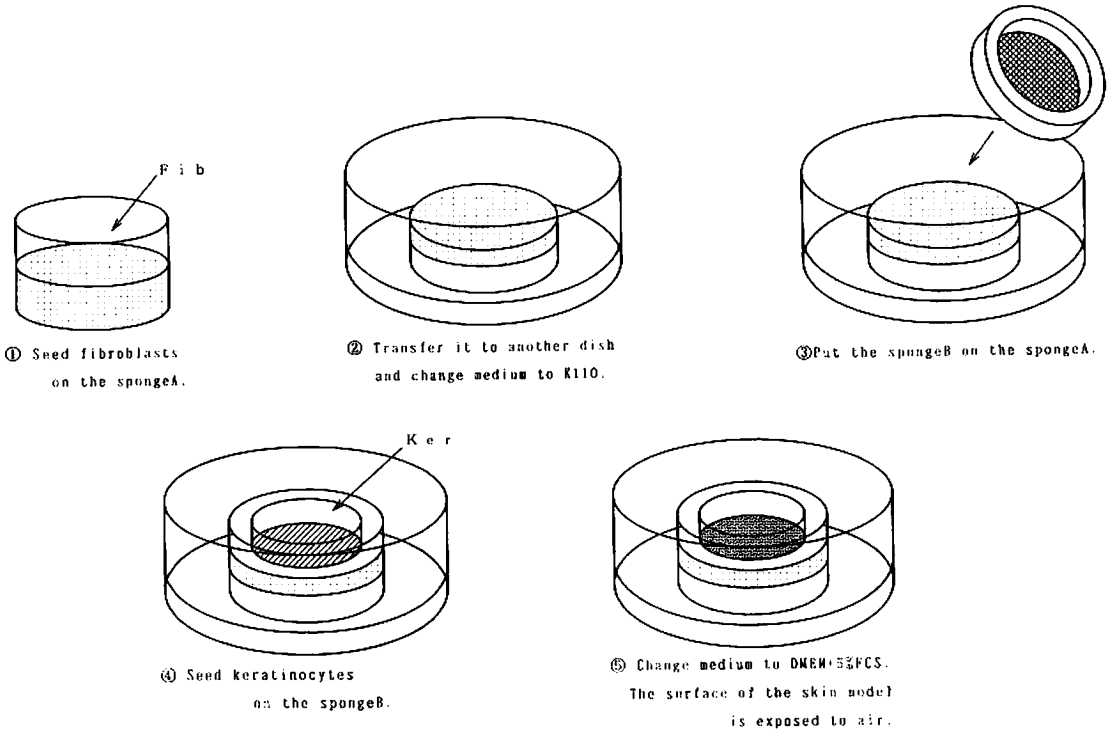

Fig. 1. The procedure to prepare the skin model.

Inc., Osaka, Japan) and have different pore sizes and cross-links. Collagen sponge A has larger pores (average pore size is 90 μm) and stronger cross-link with glutaraldehyde; sponge B has smaller pores (average pore size is 15 μm) and weaker cross-link with dehydrothermal treatment. Fig. 1 shows the procedure for preparing the skin model. Human dermal fibroblasts (4.5×10^5 cells) suspended in 0.5 mL of DMEM supplemented with 10% FBS (DMEM + 10% FBS) were seeded on to sponge A (12 mm in diameter). After 24-hour incubation, fibroblasts had attached to the sponge and the skin models were transferred to another dish. The medium was removed and replaced with K110 medium in preparation for keratinocyte attachment. Collagen sponge B, 8 mm in diameter and fixed on a plastic ring, was placed on sponge A. Human epidermal keratinocytes (4.5×10^5 cells) suspended in 0.1 mL of K110 medium were then seeded on to sponge B. After 24-hour incubation, the keratinocytes had attached to sponge B. DMEM supplemented with 5% FBS (DMEM + 5% FBS) was used for the culture medium to induce keratinocyte differentiation. At this time, the surface of the skin model was exposed to air (air-liquid interface culture method). The culture medium was changed every other day for several days.

Evaluation of skin irritancy

Each test chemical was dissolved or suspended in PBS or olive oil to achieve a 10% concentration (w/w), and 100 μL was applied to the surface of the skin models. As a control, PBS was used. After 10 minutes of exposure, skin models were washed with PBS, immersed in DMEM + 5% FBS and cultured for an additional 15 hours at 37℃ in 5% CO_2. After chemical exposure and subsequent incubation period, the skin models themselves were used for the MTT and NR assays and the supernatants were used for the LDH, IL-1α, IL-8,

and PGE_2 assays. Each assay was performed three times and the results averaged.

Time transition of skin irritancy

Each test chemical was applied to the skin model as described above. Following washing, the skin model was incubated in DMEM + 5% FBS for 3, 6, 9, 12, 18, 21, and 24 hours at 37 ℃ in 5% CO_2. Time transition of skin irritancy was examined by the MTT, IL-8, and PGE_2 assays.

Endpoints

The MTT assay was performed following the method reported by Mosmann with slight modification (Mosmann, 1983). The skin models were incubated in 1 mL of DMEM + 5% FBS containing 0.5 mg of MTT for an additional 3 hours at 37℃ in 5% CO_2. "Living cells" were dyed violet by the MTT reagents. After the skin models were washed with PBS, acidified isopropanol (isopropanol containing 0.04 N HCl) was used as an extract solvent. The absorbance of the extracts was measured at 570 nm using a UV-VIS spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan).

The NR assay was performed following the method reported by Borenfreund and Puerner with slight modification (Borenfreund and Puerner., 1985). The skin models were incubated in 1 mL of DMEM + 5% FBS containing 50 μg of NR for an additional 3 hours at 37℃ in 5% CO_2. "Living cells" were dyed reddish by taking up NR molecules. The skin models were rinsed carefully with PBS to remove remaining dye in the collagen sponge. After washing, the skin models were immersed in 1 mL of an aqueous solution of 0.5% formaldehyde containing 1% calcium chloride for 1 minute. The dyes taken up by the cells were extracted with 1% acetic acid in a mixture of ethanol/water (50/50 by volume). The absorbance of the extracts was measured at 540 nm using a UV-VIS spectrophotometer (UV-160A, Shimadzu). The cytotoxicity determined by the MTT or NR assay methods is expressed as follows:

$$\text{Cytotoxicity} = \left(1 - \frac{A_t - A_b}{A_c - A_b}\right) \times 100\,(\%) \quad (1)$$

where A_t and A_c are absorbancies of the extracts when test chemicals and PBS were applied to the skin model, respectively. A_b is the value obtained for a blank test using collagen sponge without cells.

The collected supernatant was diluted appropriately and used for the LDH, IL-1α, IL-8, and PGE_2 assays. LDH activity was determined by employing the LDH-Cytotoxic Test Wako. IL-1α, IL-8, and PGE_2 con-centrations were determined by enzyme immunoassay using Human IL-1α ELISA Kit, Human IL-8 ELISA Kit, and PGE_2 Enzyme Immunoassay Kit, respectively.

In vivo animal testing

Japanese white rabbits were used for animal testing. Each test chemical was applied by the closed patch method (Federal Resister, 1978). 24-hours after application, remaining test chemical was washed off, and then 1, 24, and 48 hours later, the skin irritancy score according to the Draize standard was evaluated.

Results

Skin model

Our skin model is constructed of two types of normal human cells, fibroblasts and keratinocytes, and collagen sponge (Fig. 2). The two divided layers resemble dermis and epidermis, as in real human skin. In the dermis counterpart, fibroblasts extend three-dimensionally within the collagen lattice. Keratinocytes form a multilayered epithelium that is similar to epidermis *in vivo*. The cells are cuboidal in the lowermost layer, becoming gradually flattened as they move up to the outer layers, and finally became enucleated. There is cornified material in the uppermost

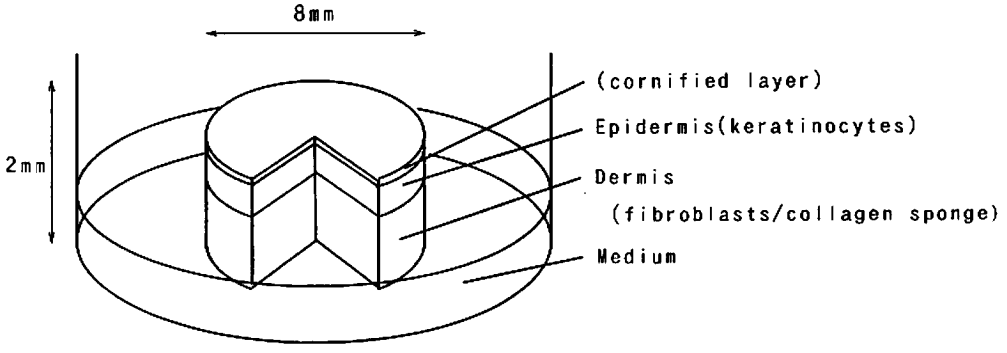

Fig. 2. Structure of the skin model.

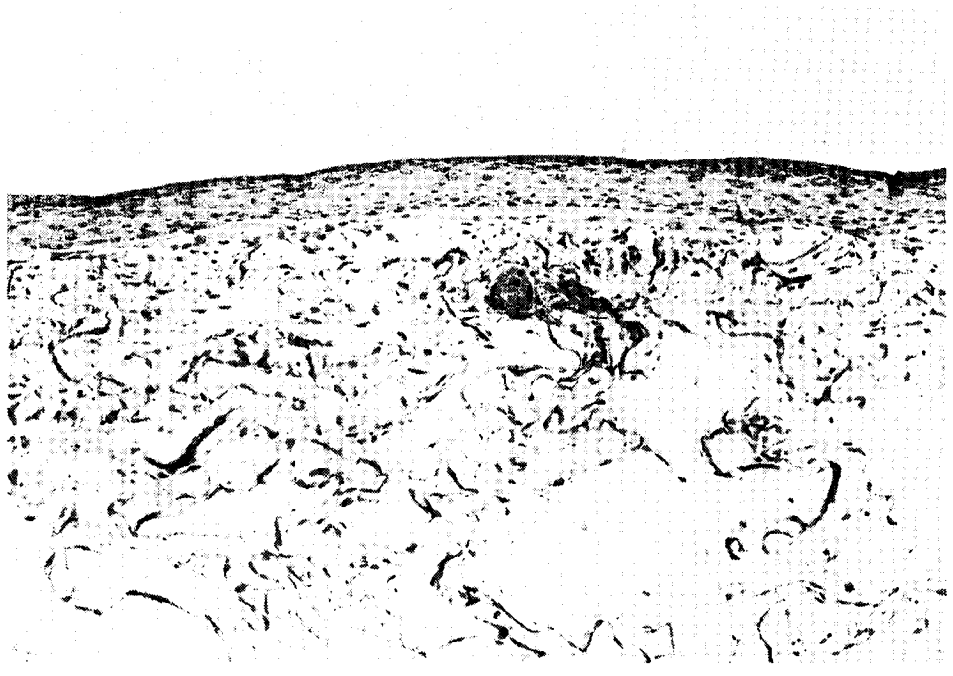

Fig. 3. Histochemical appearance of the skin model (HE staining, ×100).

layer, and keratohyaline granules are formed in the upper layers just below the cornified layers (Fig. 3)

Comparison among endpoints and in vivo animal testing

Results for each endpoint and *in vivo* animal testing are shown in Table 1, and correlation among them are shown in Table 2. Fig. 4 shows comparison between each endpoint and *in vivo* score. Compared with *in vivo* score, both the MTT and NR assays revealed good correlation, except for croton oil. On the other hand, both the IL-8 and PGE_2 assays could detect the irritancy of croton oil. On the whole, the LDH, IL-1α, IL-8, and PGE_2 assays had poor correlation with *in vivo* score. Fig. 5 shows comparison between the MTT and NR assays, the LDH and IL-1α assays, and the IL-8 and PGE_2 assays. Compared with each other,

Table 1. Results for each endpoint and *in vivo* score.

Test chemicals	*In vivo*	MTT [a)]	NR [a)]	LDH [a)]	IL-1α [b)]	IL-8 [b)]	PGE_2 [b)]
BTC	4.0	96.5	79.0	99.3	1069	90	1211
STAC	3.3	83.2	56.1	100.1	900	205	1211
SLS	6.0	100.0	98.7	-32.7	129	16	126
PL	5.3	67.6	79.7	37.6	508	131	74
TW20	0.0	-9.8	8.8	3.8	265	89	189
TW80	0.3	11.9	16.1	5.4	198	89	163
TBC	8.0	100.0	95.5	67.0	665	84	4800
DNCB	7.7	97.5	100.0	83.7	488	26	22
Croton oil	5.0	-2.7	32.5	9.6	271	367	3789
Olive oil	0.0	3.0	0.0	2.6	165	134	134

a) Percent relative values to 0% value when PBS was applied to the skin model.
b) Percent relative values to 100% value when PBS was applied to the skin model.

Table 2. Correlations among endpoints and *in vivo* score.

	In vivo						
MTT	0.827 [a)]	MTT					
NR	0.937 [a)]	0.883	NR				
LDH	0.135	0.217	0.070	LDH			
IL-1α	0.097	0.363	0.197	0.849	IL-1α		
IL-8	0.013	0.221	0.160	0.037	0.001	IL-8	
PGE_2	0.203	0.035	0.062	0.039	0.068	0.787 [b)]	PGE_2

a) Except croton oil.
b) Except TBC.

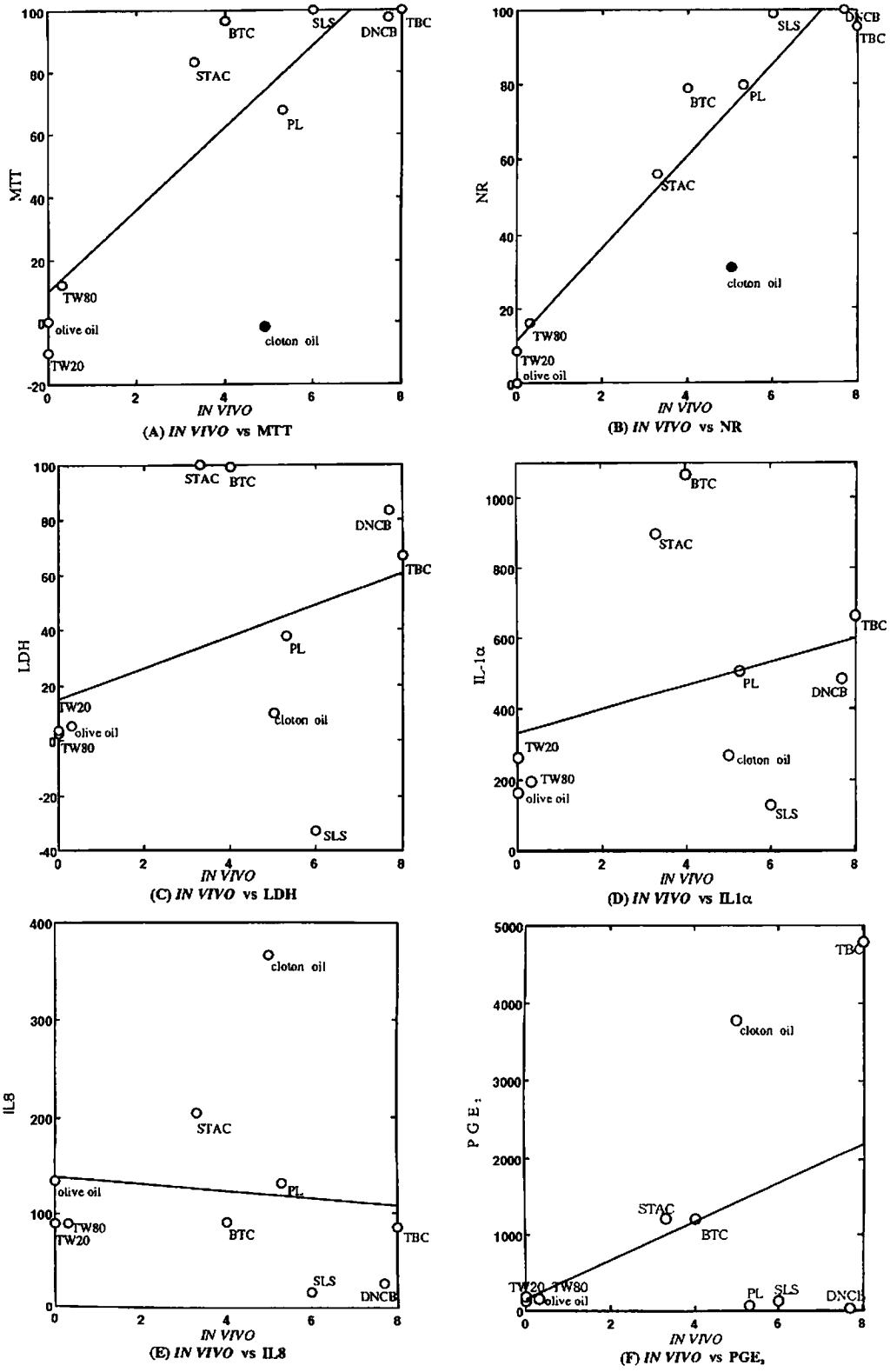

Fig. 4. Correlations between *in vivo* score and each endpoint. (A) *in vivo* vs MTT, (B) *in vivo* vs NR, (C) *in vivo* vs LDH, (D) *in vivo* vs IL-1α, (E) *in vivo* vs IL-8, and (F) *in vivo* vs PGE_2.

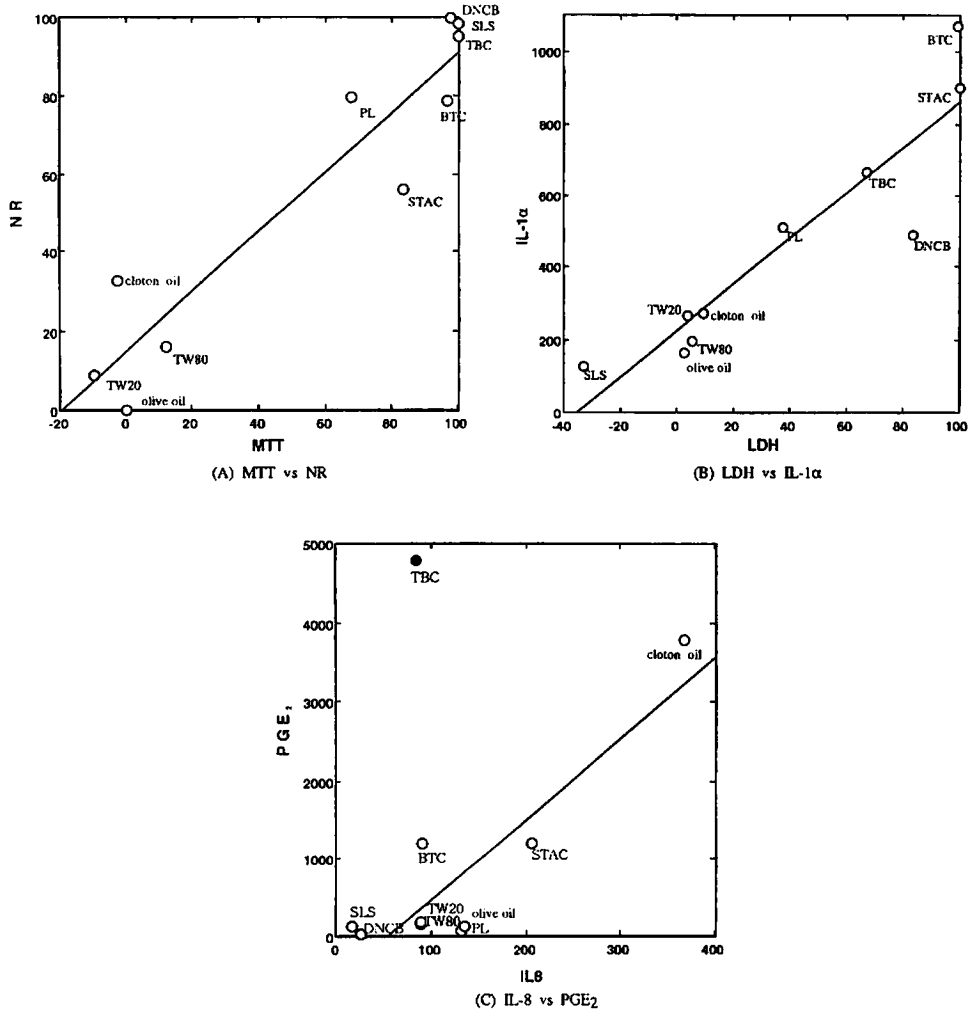

Fig. 5. Correlations between (A) MTT and NR assays, (B) LDH and IL-1α assays, and (C) IL-8 and PGE_2 assays.

the MTT and NR assays, the LDH and IL-1α assays, and the IL-8 and PGE_2 assays revealed good correlation.

Time transition of skin irritancy

It is known that IL-8 and PGE_2 are produced by cells after stimulation. To clarify the mechanism of IL-8 and PGE_2 releases, time transitions of cell viability determined by the MTT assay, and IL-8 and PGE_2 releases were examined. Croton oil, SLS, and TBC were selected for this evaluation. IL-8 and PGE_2 releases were induced by croton oil, but SLS did not induce IL-8 and PGE_2 releases in spite of its severe irritancy. In the case of TBC, there was a striking difference between IL-8 and PGE_2 releases. These results are shown in Fig. 6. After SLS apprication, cell viability decreased immediately, and both IL-8 and PGE_2 were not produced during the 24-hour incubation. After TBC application, cell viability decreased gradually and PGE_2 production

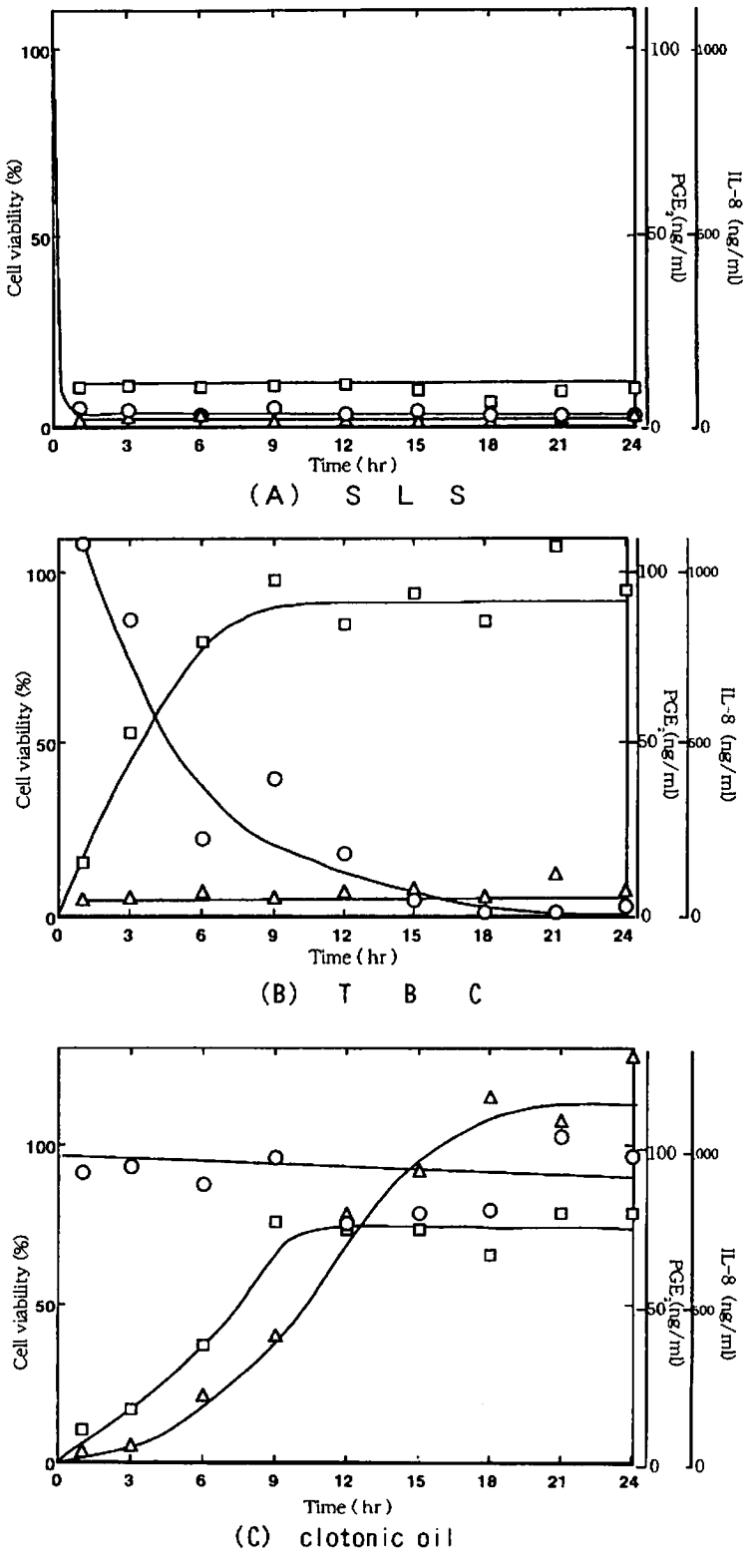

Fig. 6. Time transitions of (○) cell viability determined by MTT assay and (△) IL-8 and (□) PGE_2 releases after (A) SLS, (B) TBC, and (C) croton oil applicaton.

had begun after 3 hours, but IL-8 was not produced within 24 hours. In regard to croton oil, cell viability never decreased, and both PGE_2 and IL-8 were produced. In this case, PGE_2 production began first and IL-8 production followed soon after.

Discussion

In this study, we compared six endpoints with *in vivo* animal testing and evaluated correlation between endpoints. They could be divided into 3 groups, MTT/NR, LDH/IL-1α, and IL-8/PGE_2.

The MTT assay evaluates cell viability by measurement of mitochondrial dehydrogenase activity (Mosmann, 1983). On the other hand, the NR assay evaluates cell viability by determining the amount of NR dye which accumulates in lysosomes (Borenfreund and Puerner., 1985). The principles of these assays are different, but they equally evaluate "cell viability" and "cytotoxicity". Both assays revealed good correlations with *in vivo* animal testing, and they have the advantages of easy use and low cost compared with other endpoints. We consider that they should be tried first when testing a chemical for irritancy. However, not all irritants are cytotoxic. In the present study, croton oil, which is known as a severe irritant was not detected by the MTT and NR assays. Thus it appears that the skin irritancy of croton oil is not related to mechanisms nor linked to cytotoxicity. It is impossible to detect such types of irritancy by using the MTT and NR assays.

Both the IL-8 and PGE_2 assays could detect the irritancy of croton oil; IL-8 and PGE_2 are known as an inflammation mediators. The irritancy of croton oil is probably caused by inducing these mediators. Wilmer et al. showed that croton oil induced the release of IL-1α, IL-8, tumor necrosis factor-α (TNF-α), and granulocyte/macrophage colony stimulating factor (GM-CSF) in cultured human keratinocytes. These workers further indicated that determining the kinetics of production and the regulation of inflammation mediators in the skin will be of value in predicting various toxicities caused by chemicals (Wilmer et al., 1994). Fig. 6 shows the time dependence of IL-8 and PGE_2 production after SLS, TBC, and croton oil applications. With SLS, most of the cells died immediately, and neither IL-8 nor PGE_2 were produced. In the case of TBC, cell viability decreased gradually, and PGE_2 was produced during this period. On the other hand, most of cells were dead before the commencement of IL-8 release. These results may be due to the difference in the time required to start mediator generation after stimulation. In the case of croton oil, it is interesting that most of the cells are still alive and can produce both mediators. Because of such characteristic, some cytotoxic chemicals can be evaluated as false-negative by using IL-8 and PGE_2 assays. Where severe cytotoxic chemicals were applied, both IL-8 and PGE_2 were not produced since most of the cells had already died. In this study, SLS and DNCB were evaluated as false-negative.

Both LDH and IL-1α are substances that are stored inside cells and released by destruction of the cell membrane. Therefore, detection of these substances basically indicates cytotoxicity. However, in this study, these assays showed no correlation with the MTT and NR assays nor with the *in vivo* animal testing. Some chemicals such as SLS are known to inhibit the reaction between LDH and the detecting reagent (data not shown), and this may be one of several reasons for this lack of correlation.

The six endpoints used in this study, which were divided into 3 groups, have their own characteristics. Considering these characteristics and the assay combinations, it is possible to confidently predict skin irritancy. For example, the combination of the MTT and PGE_2 assays would be effective whereby chemicals determined to be non-cytotoxic by the MTT assay would be then tested in the PGE_2 assay. At the same time, it is possible to research

mechanisms of irritancy with a combination of some of the proposed endpoints. It is expected that the skin model will be very useful for purposes of predicting *in vivo* skin irritancy of chemicals and for studying mechanistic aspect of such irritancy.

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