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Comparison of Sensitivity Between Balb/c 3T3 Cell and HaCaT Cell by NRU Assay to Predict Skin Phototoxicity Potential

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ABSTRACT : *In order to find out the appropriate in vitro method for high correlation with in vivo, we compared the sensitivities of phototoxicity (PT) in vitro method between in human keratinocytes, HaCaT cells and in 3T3 fibroblast cells derived from Balb/c mice. Both cells were exposed to six known phototoxic chemicals : promethazine, neutral red, chlortetracycline, amiodarone, bithionol, 8-methoxypsoralen, or non-phototoxic chemical, ALS (ammonium laureth sulfate) and then irradiated with 5 J/cm^2 of UVA. Cell viability (IC_{50}) was measured by neutral red uptake (NRU) assay. The ratio of IC_{50} value of chemicals in the presence and absence of UVA was determined by the cut-off value. The phototoxic potential of test chemicals in NRU assay was determined by measuring the photoirritation factor (PIF) with a cut-off value of 5. In both 3T3 and HaCaT cells, all known phototoxic chemicals were positive (over 5 of PIF value), except that bithionol was found to be non-phototoxic to HaCaT cells, and ALS, non-phototoxic chemical was negative. These results suggest that Balb/c 3T3 cell was more sensitive than HaCaT cell to predict phototoxicity potential.*

Key Words : *3T3 fibroblast, HaCaT cell, NRU, Phototoxicity, Sensitivity*

I. INTRODUCTION

Evaluation of the phototoxic potential of new products or ingredients prior to human testing is generally performed on animals. However, as well as ethical and financial objections to these methods, there are also scientific drawbacks such as intra- and interlaboratory variability and the fundamental difference between animal and human in morphological aspects of skin. Therefore, simple and reproducible *in vitro* tests are required as a screening procedure to estimate the phototoxicity of chemicals. One of these techniques, *in vitro* photoirritant assay using cultured Balb/c 3T3 cells has given promising results for the screening of photoirritants (Dixit *et al.*, 1994; Liebsch *et al.*, 1994; Spielmann *et al.*, 1994a, b, 1998a, b).

An advantage of human skin cultures is that they are relevant to the organ (integument) and species of concern and as such are mechanistically sound alternatives to traditional skin irritation test (Lee *et al.*, 1999, 2000a). So, there is a need to compare the sensitivity between the human keratinocyte, HaCaT and 3T3 cell to predict skin phototoxicity potential. The neutral red uptake (NRU) assay has been used extensively to study the toxic effects of chemicals on a variety of different cell types grown in monolayer cultures (Borenfreund and Puerner, 1986; Lee *et al.*, 1999) and used as an alternative method to replace the animal test for screening of phototoxicants (Spielmann and Liebsch, 2001).

The aim of this study is to make a comparison the sensitivity between the HaCaT and 3T3 cell to predict skin phototoxicity potential by testing several known phototoxic chemicals (promethazine, neutral red, chlortetracycline, amiodarone, bithionol, 8-methoxypsoralen) and non-phototoxic substance (ammonium laureth sulfate).

II. MATERIALS AND METHODS

1. Chemicals

Dulbecco's modified eagle's medium (DMEM), fetal

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bovine serum (FBS), penicillin/streptomycin, Dubelcco's phosphate buffered saline (PBS), trypsin-EDTA and trypsin were obtained from Gibco (USA). On the basis of information from the literature (Spielmann *et al.*, 1998a, b), the following chemicals were selected for testing : Phototoxicant - promethazine, neutral red, chlortetracycline, amiodarone, bithionol, 8-methoxypsoralen (Sigma, USA), and Non-phototoxicant - ammonium laureth sulfate (Pacific, Korea). All chemicals were dissolved in EBSS (Earles balanced salt solution).

2. Cell culture

The HaCaT cell was kindly provided by Professor Fusenig (German Cancer Research Center (DKFZ), Boukamp *et al.*, 1988). Cells were cultured in a 5% CO_2 atmosphere at 37°C in DMEM with 10% FBS, 2 mM glutamine, 20 mM sodium bicarbonate and antibiotics. Balb/c 3T3 clone A31 cells were obtained from ATCC (American Type Culture Collection) and were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 20 mM sodium bicarbonate and antibiotics.

Cell density was adjusted to 10^5 cells/ml into 96 well tissue plate and cells were incubated for 24 hrs before cytotoxicity assay.

3. UV light source and dosimetry

UV irradiation was carried out using UV lighter (RMX 3 W lighter, Dong Sung Lab. Tech., Seoul, Korea) equipped with a F 40 M UVA lamp, emitting a wavelength 300~400 nm (Vilbert-Lourmat, Merne-La-Vallee Cedex 2, France). The radiation intensity was monitored by a VLX-3W radiometer (Vilbert-Lourmat) equipped with VLX-312 (UVB) sensors responding at 365nm. In a preliminary study, we confirmed both human and mice fibroblasts cells met the quality criteria if at a UVA dose of 5 J/cm^2 viability was not reduced more than 10%.

4. Experimental desgin

Both HaCaT and 3T3 Balb/c cells were cultured in DMEM in 96-well microtitre plates. After 24 hr the medium was removed, the cells were washed twice in EBSS and eight concentrations of the test chemicals dissolved in EBSS were added. Test chemicals that were insoluble in EBSS were dissolved in dimethyl sulfoxide (DMSO) before use and added at a maximum of 1% DMSO in EBSS. After 1hr of preincubation with the test chemicals the plates were exposed to UVA (5 J/cm^2). During this period the second set of plates with the same chemicals was kept in the dark. After light exposure EBSS was replaced by DMEM (without any test chemicals) and NRU was determined 24 hr later.

5. NRU assay

The NRU assay was performed according to the method of Borenfreund and Puerner (1985). To each well was added 0.2 *ml* medium containing 50 μg NR/*ml*. The plate was returned to the incubator for another 3 hours to allow for the uptake of the vital dye into the lysosomes of viable uninjured cells. Thereafter the medium was removed, the cells were washed rapidly with a mixture of 1% formaldehyde-1% $CaCl_2$ and then 0.2 *ml* of a solution of 1% acetic acid-50% ethanol was added to each well to extract the dye. After short agitation on a microtitre-plate shaker, the plate was transferred to a microplate reader (E-max, USA) equipped with a 540 nm filter to measure absorbance of the extracted dye.

6. Evaluation of *in vitro* data and statistical analysis

The cytotoxicity of each chemical was expressed in terms of its IC_{50}, i.e. the concentration of test agent that causes the 50% inhibition of growth, as compared with untreated control cells. IC_{50} values were calculated from the dose-response curves by non-linear regression analysis. The values obtained in the light and dark experiments were compared by calculating a PIF(photoirritation factor) according to Spielmman *et al.* (1998a). $PIF = IC_{50}(-UV)/IC_{50}(+UV)$. The cut-off value of the factor to discriminate between photoxicants and non-photoxicants was calculated using a discriminant analysis of the results obtained in the prevalidation study (Spielmmann *et al.*, 1998a), which revealed a cut-off value of $PIF \geq 5$ for predicting phototoxic potential.

III. RESULTS

1. *In vitro* cytotoxicity of chemicals

The dose-response relationship was established for all test substances, and IC_{50} values of each chemical was calculated from these dose-response plots. The IC_{50} values of chemicals in Balb/c 3T3 fibroblast cell were determined by means of NRU assay (Figs. 1, 2 and Table 1).

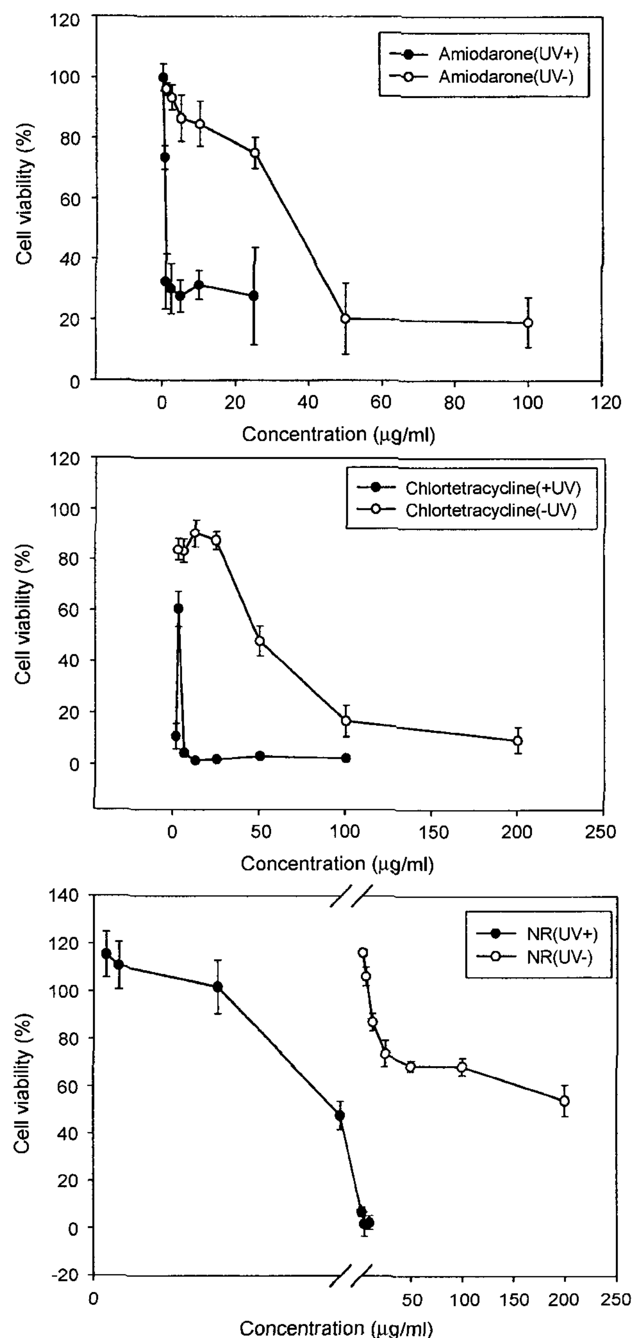

Fig. 1. *In vitro* cytotoxic effects of amiodarone, chlortetracycline and neutral red for 1 hour exposure on 3T3 cells using neutral red uptake assay.

Table 2 showed the IC_{50} values (µg/m*l*) of chemicals in HaCaT cell determined by means of NRU assay. The IC_{50} vaules of amiodarone and that of neutral red in HaCaT cells with UVA exposure were observed, *803.63 µg/ml and 464.20 µg/ml respectively,* but the values of both chemical in HaCaT cells without UVA was not determined.

2. Phototoxicity of test chemicals in HaCaT and 3T3 cell

The PIF values of Balb/c 3T3 cells could be determined in all chemicals. Table 1 showed that all of 6 known phototoxicants (Amiodarone, chlortetracycline, 8-MOP, neutral red, promethazine, and bithionol), and one of non-phototoxicant, ALS were correctly identi-

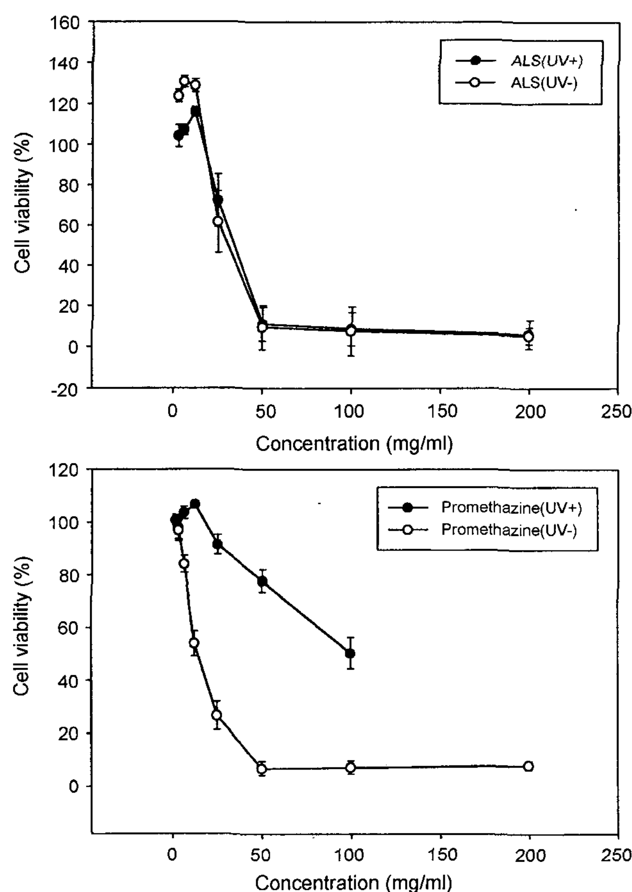

Fig. 2. *In vitro* cytotoxic effects of ALS and promethazine for 1 hour exposure on 3T3 cells using neutral red uptake assay.

Table 1. Phototoxicity of 7 chemicals in the 3T3 Balb/c fibroblast cell NRU cytotoxicity assay

No	Chemical	Phototoxicity *in vivo**		3T3 fibroblast NRU cytotoxicity[†]				
		Animals	Humans	Mean IC_{50} UV (μg/ml)	Mean IC_{50} UV (μg/ml)	PIF -UV/+UV	N	Result
1	Amiodarone	+	+	29.81	1.54	19.4	6	+
2	Chlortetracycline	+	+	40.23	3.71	10.8	6	+
3	8-MOP	+	+	57.72	1.62	35.6	6	+
4	Neutral red	+	+	194.81	16.21	12.0	6	+
5	Promethazine	+	+	432.57	8.85	48.9	6	+
6	Bithionol	s+/t-	+	0.07	0.41	5.9	6	+
7	ALS	+	+	27.16	41.95	0.7	6	-

8-MOP (8-methoxypsoralen), ALS (ammonium laureth sulfate).
**In vivo data* cited from Spielmann *et al.* (1998a).
+ = phototoxic, - = non-phototoxic, +/- = inconclusive, PIF = Photoirritation factor, s = systemic application, t = topical application.
[†]Means are arithmetic means for the number of calculations (n) shown (standard deviations are not shown).
§ = No IC_{50} could be determined.

Table 2. Phototoxicity of 7 chemicals in the HaCaT cells NRU cytotoxicity assay

No	Chemical	Phototoxicity *in vivo**		HaCaT cell NRU cytotoxicity[†]				
		Animals	Humans	Mean IC_{50} UV (μg/ml)	Mean IC_{50} UV (μg/ml)	PIF -UV/+UV	N	Result
1	Amiodarone	+	+	§	803.63	§	6	+
2	Chlortetracycline	+	+	131.80	37.10	3.6	6	-
3	8-MOP	+	+	10.27	1.22	8.4	6	+
4	Neutral red	+	+	§	464.20	§	6	+
5	Promethazine	+	+	117.70	0.06	1,880	6	+
6	Bithionol	s+/t-	+	9.70	7.80	1.2	6	-
7	ALS	+	+	21.30	30.80	0.7	6	-

8-MOP (8-methoxypsoralen), ALS (ammonium laureth sulfate).
**In vivo data* cited from Spielmann *et al.* (1998a).
+ = phototoxic, - = non-phototoxic, =/- = inconclusive, PIF = Photoirritation factor, s = systemic application, t = topical application.
[†]Means are arithmetic means for the number of calculations (n) shown (standard deviations are not shown).
§ = No IC_{50} could be determined.

fied with the assay.

However, the PIF values of HaCaT cells could be determined in chlortetracycline, neutral red, promethazine, bithionol and ALS since amiodarone and 8-MOP were not cytotoxic to HaCaT cells in dark (UVA) even at the highest concentration (Cmax) tested. Therefore we classified the phototoxic potential as below according to Spielmman *et al.* (1998a). If Cmax (-UVA) and IC_{50} (+UVA) were determined, PIF > 1 is phototoxic. Table 2 showed that 4 of 6, known phototoxicants and ALS were correctly identified with the assay. In the case of bithionol and chlortetracycline, the phototoxicity potential was negative since PIF value was 1.2, and 3.6 respectively.

IV. DISCUSSION

The purpose of this investigation was to compare the sensitivities between in the human keratinocytes, HaCaT and in 3T3 cells for prediction of skin phototoxicity potential by testing several known phototoxic chemicals (promethazine, neutral red, chlortetracycline, amiodarone, bithionol, 8-methoxypsoralen) and non-phototoxic substance (ammonium laureth sulfate). Because *in vitro* systems using human keratinocytes have the advantage, that they simulate mores aspects of *in vivo* human skin than cell lines and cells of other tissues and species (Maier, 1994). Unexpectedly, four of 6 known phototoxicants were correctly identified in HaCaT cells (Table 2), however all of 6 known phototoxicants were correctly identified in Balb/c 3T3 fibroblasts (Table 1).

The results from this study using 3T3 NRU PT assay was more correctly identified than the results using human fibroblast NRU PT assay from our previously report (Lee *et al.*, 2000b). In a recent EC/

COLIPA validation trial of *in vitro* methods for phototoxicity testing, the NRU growth inhibition assay using Balb/c 3T3 fibroblast to determine the cytotoxicity was adapted for phototoxicity testing (Spielmann *et al.*, 1998a, b, 2001). Their reports suggested that the 3T3 NRU phototoxicity test assay is able to identify the phototoxic as well as the non-phototoxic chemicals. Therefore, this investigation could support that 3T3 NRU PT test was well suited to identifying the phototoxic potential of chemicals.

Bithionol was found to be non-phototoxic to HaCaT cells in this experiment (Table 2). Clothier *et al.* (1999) reported that bithionol was consistently found to be non-phototoxic to normal human primary keratinocytes, although the same chemical was shown to have clear phototoxic effects with 3T3 cells. They suggested these results could be due to a mouse human difference, or a fibroblast/keratinocyte difference. Okamoto *et al.* (1999) reported that bithionol did neither produce singlet oxygen nor react to histidine. Reid *et al.* (2001) reported that bithionol alone reduced the amount oxidative stress in normal human keratinocytes, while following photoactivation, an augmentation in the amount of oxidative stress and cell cytotoxicity was observed. They explained bithionol might stimulate the ability of human keratinocyte to quench free radicals in response to the addition of bithionol, by intracellular antioxidant, scavenger, defense mechanism. We guess our result would be in part explained by the suggestion of Reid *et al.* (2001) even though the cell is immortalized.

In this study, 3T3 cell was more sensitive than human keratinocyte, HaCaT cell to predict phototoxicity potential. These results strongly suggested that 3T3 NRU PT test was well suited to identifying the phototoxic potential of chemicals. Further experiments are necessary with special attention paid to mechanism study of false negative in human skin cells, and further study of *in vitro* phototoxicity alternatives using three-dimensional human skin analogues and its pre-validation trials.

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