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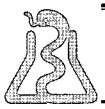

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Validation of Photo-comet Assay as a Model for the Prediction of Photocarcinogenicity

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ABSTRACT. Recent reports on the photocarcinogenicity and photogenotoxicity of many compounds led to an increasing awareness for the need of a standard approach to test for photogenotoxicity. The comet assay has been recently validated as a sensitive and specific test system for the quantification of DNA damage. Thus, the objectives of this study are to investigate the utility of photo-comet assay for detecting photo-mutagens, and to evaluate its ability to predict rodent photocarcinogenicity. Photo-comet assays were performed using L5178Y $Tk^{+/-}$ mouse lymphoma cells on five test substances (8-methoxypsoralen, chlorpromazine, lomefloxacin, anthracene and retinoic acid) that demonstrated positive results in photocarcinogenicity tests. For the best discrimination between the test substance-mediated DNA damage and the undesirable DNA damage caused by direct UV absorption, a UV dose-response of the cells in the absence of the test substances was firstly finalized. Out of 5 test substances, positive comet results were obtained for chlorpromazine, lomefloxacin, anthracene and retinoic acid while 8-methoxypsoralen found negative. An investigation into the predictive value of this photo-comet assay for determining the photocarcinogenicity showed that photo-comet assay has relatively high sensitivity. Therefore, the photo-comet assay with mammalian cells seems to be a good and sensitive predictor of the photocarcinogenic potential of new substances.

Keywords: Photogenotoxicity, Photo-comet assay, Photocarcinogenicity.

INTRODUCTION

In recent years attention has been called upon the fact that toxic intermediates that are generated upon photo-activation of a substance can also lead to DNA damage. Such damage may lead to mutated skin cells, which in turn can contribute to an elevated skin cancer risk. Common drugs known to cause sunlight related adverse reactions include psoralens, phenothiazines (anti-depressants, such as chlorpromazine), non-steroidal anti-inflammatories, and quinolenes (both the non-fluorinated nalidixic acid and fluorinated quinolones). Psoralen combined with exposure to ultraviolet A (PUVA) therapy for psoriasis, which uses 8-methoxypsoralene (8-MOP) in conjunction with UVA radiation, was shown to increase the risk of skin cancer in patients (Stern *et al.*, 1979). Retin-A, a form of trans-retinoic acid, was found to cause skin cancer in an animal model system using hairless mice, which includes exposure to stimulated sunlight (Davies and Forbes, 1988). Phenothiazines, such as chlorpromazine, compazine, and perphenazine, are photomutagenic, but only chlorpromazine has been tested for photocarcinogenicity (Jose, 1979; Kelly *et al.*, 1989; Kelly-Garvert and Legator, 1973).

However, photocarcinogenicity test, which is usually determined using hairless mice, is time-consuming and costly with many animals. Thus, it would be useful to have short-term photogenotoxicity tests that are predictive of drug enhancement of UV-induced skin carcinogenesis. Photogenotoxicity is a genotoxic response, which is observed after exposure to a chemical and a (non)-genotoxic dose of light/UV radiation (Spielmann *et al.*, 2000). The short-term *in vitro* assays such as the Ames test, chromosomal aberration (CA) test and mouse lymphoma assay (MLA) are widely used to determine the genotoxic potential for new chemical entities during a pre-clinical safety evaluation. In the past

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few years some effort has been put into the evaluation for such systems, in particular standard test protocols have been generated for the *in vitro* photo-micronucleus test and photo-CA assay with Chinese hamster cells. Until now, results indicated that photogenotoxicity assays did not necessarily predict effects in photocarcinogenicity studies in mice (Jacobs *et al.*, 1999).

Recently, a more useful approach called comet assay (single cell gel electrophoresis), which is a rapid and sensitive fluorescence microscopic method for detection of primary DNA damage on the individual cell level (Olive *et al.*, 1990; Singh *et al.*, 1988), is increasingly used to evaluate the genotoxicity of test substances. In comet assay, cells with increased DNA damage display an increased migration of genetic material in the direction of the electrophoresis. The extent of DNA damage is quantified by measuring the displacement of the genetic material between the cell nucleus and the resulting tail. Compared with other genotoxicity assays, the advantages of comet assay include the relatively short-time period needed to complete an experiment, the sensitivity for detecting low levels of DNA damage, and use as a potentially high-throughput screening assay (Tice *et al.*, 2000)

The aim of present study was therefore to perform the photo-comet assay with the known photocarcinogenic substances and to compare the photo-comet assay results with their reported photocarcinogenicity results using the alkaline (pH > 13) version of comet assay (Singh *et al.*, 1988) accepted as the optimal version of the assay by expert panel at the International Workshop on Genotoxicity Test Procedures (IWGTP) (Tice *et al.*, 2000). Photo-comet assay was performed on five test substances: 8-methoxypsoralen (8-MOP, photoactive substance that forms DNA adducts in the presence of ultraviolet A irradiation), chlorpromazine (an aliphatic phenothiazine; an alpha-adrenergic blocking agent), lomefloxacin (an antibiotic in a class of drugs called fluoroquinolones), anthracene (a tricyclic aromatic hydrocarbon; a basic substance for production of anthraquinone, dyes, pigments, insecticides, wood preservatives and coating materials) and retinoic acid (a retinoid compound closely related to vitamin A). Overall the data indicate that photo-comet assay has the strong association with photocarcinogenicity and is suitable screening system for predicting photocarcinogens.

MATERIALS AND METHODS

Chemicals

The test compounds used for photo-comet assay in this study were retinoic acid (CAS# 302-79-4), lomefloxacin (CAS# 98079-51-7), chlorpromazine (CAS# 69-09-0), anthracene (CAS# 120-12-7), and 8-methoxypsoralene (CAS# 298-81-7). All compounds were obtained from Sigma-Aldrich (St. Louis, MO). The reported photogenotoxicity and photocarcinogenicity results for each compound are presented in Table 1.

Table 1. Published photo-comet assay and photocarcinogenicity results for each test substance

Compounds	Photo-comet assay	Photo-Carcinogenicity
8-MOP	Neg[1]	Pos[2]
Chlorpromazine	Pos[1]	Pos[3]
Lomefloxacin	Pos[4]	Pos[5]
Anthracene	N/A	Pos[6]
Retinoic acid	N/A	Pos[7]

Pos, positive response; Neg, negative response; N/A, Not Available.
(1) Unpublished results by Brendler-Schwaab *et al.*, in Brendler-Schwaab *et al.*, 2004; (2) Nagayo *et al.*, 1983; (3) Kelly *et al.*, 1989; (4) Chetlat *et al.*, 1996; (5) Ball *et al.*, 1999; (6) Blackburn and Taussig, 1975; (7) Fu *et al.*, 2003.

UV irradiation

For irradiation experiment, an Vilber Lourmat 40 W black-light lamp (λ_{max} = 365 nm) (Vilber Lourmat, Marne la Valle, France) was used as light source at a distance of 15 cm. UVA and UVB doses were measured using a Radiometer RM-21 manufactured by Dr. Gröbel UV-Elektronik GmbH (Ettlingen, Germany) equipped with a UVA sensor (spectral range 315~400 nm) and a UVB sensor (spectral range 280~315 nm).

Cell line and cell culture

L5178Y $Tk^{+/-}$ mouse lymphoma cells were a gift from Toxicology Center, LG Life Sciences/Research Park (Daejeon, Korea). L5178Y $Tk^{+/-}$ mouse lymphoma cells were cultured in RPMI 1640 medium supplemented with 100 units of penicillin, 100 mg of streptomycin/Liter, 2 mM L-glutamine, and 10% horse serum (GIBCO-Invitrogen, Carlsbad, CA) at 37°C in a 5% CO_2 atmosphere.

Photo-comet assay

Exponentially growing L5178Y $Tk^{+/-}$ mouse lymphoma cells were plated at a density of 1.8×10^6 cells per 60 mm dish in 3 ml of Earle's Balanced Salt Solution (EBSS, w/o phenol red). After pretreatment of cells with each test substance for 60 min in an incubator, culture dishes with lids were then exposed to a UVA dose of 1 J/cm^2 and a UVB dose of 0.01 J/cm^2, which did not induce DNA damage and cytotoxicity. Cell viability was determined by trypan blue exclusion. For the comet assay, cells were processed immediately after the end of UV exposure. The comet assay was performed as

described by Tice *et al.* (2000) and manufacturer's instruction. Briefly, cell suspension (25 μl) was mixed 1 : 10 with 250 μl molten low melting point (LMP) agarose, and 75 μl of the mixture were rapidly spread on CometSlide™ (Trevigen, Gaithersburg, MD). After gelling for 20 min at 4°C in the dark, the slides were incubated in ice-cold lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris base, 1% sodium lauryl sarcosinate and 1% Triton X-100) at 4°C for at least 1 h to remove cellular proteins, leaving the DNA as 'nucleoids'. Slides were then washed three times with neutralization buffer (0.4 M Tris, pH 7.5) for 5 min and incubated in fresh alkaline buffer (0.3 M NaOH and 1 mM EDTA, pH > 13) for 30 min at room temperature to allow unwinding of DNA and expression of alkali-labile sites. Electrophoresis was then carried out at room temperature in fresh ice-cold alkaline electrophoresis buffer for 30 min (1 V/cm; 300 mA). All the above steps were conducted under yellow light to prevent additional DNA damage. After electrophoresis, slides were gently washed three times for 5 min in fresh neutralization buffer and exposed to 70% ethanol for 5 min. After drying at room temperature, slides were stained with 25 μl of ethidium bromide solution (20 μg/ml) and covered with a coverslip. Comets were examined at 200 × magnification using a fluorescence microscope (Nikon E600) that connected through the CCD camera to an image analysis system (Komet 5.0, Kinetic Imaging, Liverpool, UK). Hundred cells (50 cells from each of two replicate slides) were selected and analyzed for each concentration of test substances. In the selection of cells, the edges and cells around the air bubbles were avoided (Collins, 2004). The vehicle was used as negative control and H_2O_2 was used as positive control under condition without UV irradiation.

Evaluation of DNA damage

For quantitative evaluation, tail moment (tail length × % DNA in tail) was recorded. The statistical significance was calculated using the C^2-test and Fisher's exact test for comparison between the negative control and test item-treated groups, and Cochran-Armitage trend test for dose-response. Differences were regarded as statistically significant, if $P < 0.05$. The result of the study was judged as positive if there was a concentration-related result or a reproducible result.

RESULTS

Determination of UV irradiation conditions

The best discrimination between the test substance-mediated genotoxicity and the undesirable genotoxicity caused by direct DNA absorption can be achieved by selecting the suitable UV doses. The recommendation was also made by an international group to irradiate with a mixture of both UVA and UVB. Thus, a full irradiation dose-response curve of the test system was determined in the absence of test substance. The influence of the UVB component on the tail moment and the viability for irradiation with light possessing different UVA/UVB relationships in L5178Y $Tk^{+/-}$ mouse lymphoma cells was shown in Fig. 1. An increasing relative content of UVB in the spectrum resulted in an increasing the tail moment. The maximum non-mutagenic UVA/UVB dose appeared to be 1/0.01 J/cm^2 at which it was not observed the significant cell death either. Thus, photo-comet assay to detect test substance-induced photogenotoxicity was performed with a UVA/UVB doses of 1/0.01 J/cm^2.

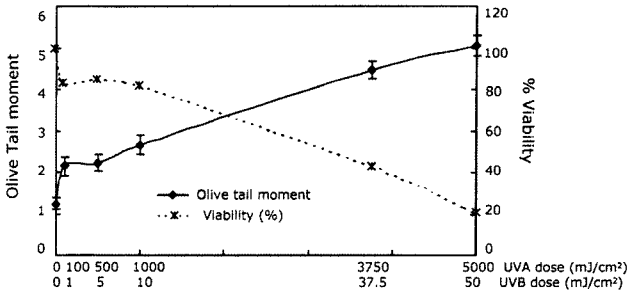

Fig. 1. Determination of UV irradiation conditions. L5178Y $Tk^{+/-}$ mouse lymphoma cells were irradiated with Vilber Lourmat 40 W black-light lamp at different UV doses. Left axis indicates the tail moment, which was determined as described in Material and Methods. Right axis indicates the viability determined by comparing viable cell counts measured through trypan blue exclusion method in test substance and negative control cultures. Data represent 100 cells/treatment group.

Photo-Comet assay

A dose range-finding test was performed to determine the highest concentration showing about 70% viability for the photo-comet assay (data not shown). After establishing positive effect in the comet assay with hydrogen peroxide (200 μM, 1 h) without UV irradiation, photo-comet assay was performed with 5 test compounds that demonstrated positive results in photocarcinogenicity test using hairless mouse model. The cytotoxic effects of 5 test substances were measured by trypan blue exclusion method. Among various tail parameters calculated by computerized image analysis (Olive *et al.*, 1990), comet assay results were presented as Olive tail moment. A major advantage of using the tail moment as the index of DNA damage is

Table 2. Photo-comet results using L5178Y $Tk^{+/-}$ mouse lymphoma cells exposed to each test substance in the presence and in the absence of irradiation from Vilber Lourmat 40 W black-light lamp

Without UV			UVA : UVB (1 : 0.01 J/cm^2)		
Conc. (μg/ml)	Olive Tail moment	Viability (%)a)	Conc. (μg/ml)	Olive Tail moment	Viability (%)
8-Methoxypsoralen (Negative)					
0	1.44 ± 0.16	100	0	1.96 ± 0.19	100
3.125	1.93 ± 0.20	99	0.313	2.48 ± 0.20	89
6.25	2.21 ± 0.17	99	0.625	2.61 ± 0.20	84
12.5	2.53 ± 0.23	89	1.25	3.21 ± 0.29	79
25	2.89 ± 0.23	69	2.5	3.17 ± 0.25	72
Chlorpromazine (Positive)					
0	1.81 ± 0.11	100	0	2.77 ± 0.16	100
0.5	3.20 ± 0.20	98	0.005	4.27 ± 0.21**	94
1	4.37 ± 0.20**	94	0.01	4.36 ± 0.22**	90
2	4.71 ± 0.27**	86	0.02	5.19 ± 0.29**	82
4	5.09 ± 0.25**	70	0.04	6.77 ± 0.32**	65
Lomefloxacin (Positive)					
0	1.91 ± 0.18	100	0	2.64 ± 0.16	100
62.5	2.87 ± 0.23	101	1	5.18 ± 0.53**	98
125	3.01 ± 0.25	100	2	15.35 ± 0.74**	89
250	3.35 ± 0.27**	81	4	20.17 ± 0.81**	83
500	4.48 ± 0.47**	70	8	35.70 ± 1.29**	79
Anthracene (Positive)					
0	1.44 ± 0.16	100	0	1.96 ± 0.19	100
0.195	2.48 ± 0.23	98	0.003	3.37 ± 0.26	84
0.391	2.90 ± 0.23*	85	0.006	4.90 ± 0.34**	82
0.781	3.45 ± 0.24**	75	0.013	5.73 ± 0.37**	80
1.563	3.37 ± 0.23**	70	0.025	8.12 ± 0.58**	75
Retinoic acid (Positive)					
0	1.44 ± 0.16	100	0	1.96 ± 0.19	100
0.063	2.47 ± 0.27	90	0.05	2.88 ± 0.21	84
0.125	2.51 ± 0.21	87	0.1	4.34 ± 0.36**	83
0.25	3.10 ± 0.28*	80	0.2	4.93 ± 0.24**	79
0.5	2.76 ± 0.21	72	0.4	8.19 ± 0.57**	71

a)Viability: % cells of control; measured by trypan blue exclusion.

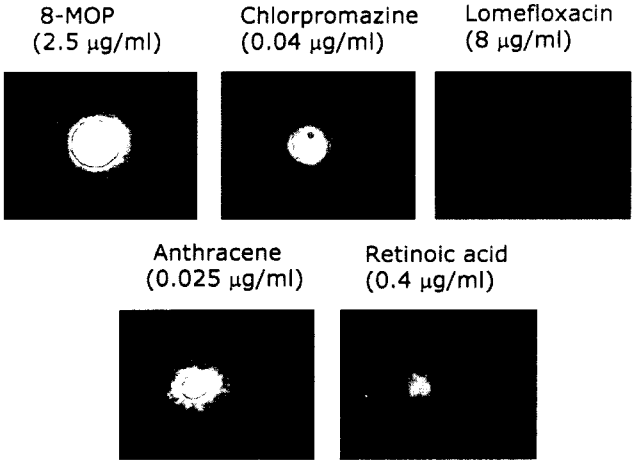

Fig. 2. The representative photomicrographs of damaged DNA observed after the treatment with test compounds showing positive response after UV irradiation in photo-comet assay.

that both the amount of damaged DNA and the distance of migration of the genetic material in the tail are represented by a single number. The results are summarized in Table 2. In particular, the representative photomicrographs of damaged DNA induced by 5 test substances showing increase in tail moment after UV irradiation are shown in Fig. 2.

8-Methoxypsoralene (8-MOP)

Consistent with the published results, 8-MOP did not induce a statistically significant increase in tail moment regardless of UV irradiation.

Chlorpromazine

Chlorpromazine caused a statistically significant increase in tail moment regardless of UV irradiation. However, after exposure to UV irradiation, DNA damage was observed at concentrations 100-fold lower than

without UV irradiation.

Lomefloxacin

Lomefloxacin caused a marked increase in tail with linear trend at the concentration of 1, 2, 4 and 8 μg/ml at cell survival of > 79% after exposure to UV irradiation. Conversely, when cells were exposed to lomefloxacin in the absence of UV irradiation, the slight increase in tail moment was noted at much higher concentrations.

Anthracene

When the cells were exposed to UV irradiation, anthracene caused a dose-related and statistically significant increase in tail moment at the concentration of 3, 6, 13 and 25 ng/ml. However, such as lomefloxacin, anthracene-induced DNA damages were detectable at much higher concentrations in the absence of UV irradiation.

Retinoic acid

Retinoic acid induced a significant increase in tail moment with linear trend at the concentration of 0.05, 0.1, 0.2 and 0.4 μg/ml after exposure to UV irradiation. Without UV irradiation, a weak but statistically significant DNA damage was observed only at 0.25 μg/ml.

Relationship between photo-comet assay and the reported photocarcinogenicity results

The reported photocarcinogenicity results for each test substance were presented in Table 1. Out of 5 positive test substances results in rodent photocarcinogenicity, four showed positive in present photo-comet assay. With this our limited dataset, an investigation into the predictive value of these short-term photogenotoxicity tests for determining the photocarcinogenicity was carried out (Table 3). Statistical analyses include sensitivity (the ability of a test to predict photocarcinogenicity). The agreement between the results of the photocarcinogenicity and photo-comet assay was found to be satisfactory on a qualitative basis. Photo-comet assay has the high sensitivity suggesting that this test would be a good predictive test to minimize false negatives.

Table 3. Relationship between outcome of photo-comet assay and the reported photocarcinogenicity results

Assay		Photo-comet assay	
		-	+
Photocarcinogenicity	-	0	0
	+	1	4
	Total	1	4
Sensitivity[a]		0.80	

The reported photocarcinogenicity results of chemical substances were compared with the results from photo-comet assay.
[a]Sensitivity: proportion of carcinogens positive in photo-comet assay.

DISCUSSION

The *in vitro* comet assay is a sensitive genotoxicity test for the detection of DNA damage and repair (Speit and Hartmann, 2006). The alkaline version of the comet assay (Singh *et al.*, 1988) detects primary (repairable) DNA single and double strand breaks and alkali-labile sites. In this study, to determine the use of *in vitro* photogenotoxicity tests for prediction of rodent/human photocarcinogenicity, *in vitro* photo-comet assay was compared for their suitability in detecting photogenotoxic compounds. Photogenotoxicity is to evaluate the genetic health of the surviving cells while phototoxicity is the measurement of acute effect of UV stress, resulting in life or death of a cell (Meunier *et al.*, 2002). Thus, to be biologically relevant, the genotoxicity endpoints evaluated through a particular methodology should only be observed in cells treated by conditions leading to no or moderate toxicity (survival > 70% of untreated controls). In this study, a dose range-finding test was performed to determine the highest concentration showing about 70% viability for the photo-comet assay. On the other hand, the OECD draft guideline for *in vitro* phototoxicity tests states that hepatic metabolic activation does not seem to play a role for the *in vitro* detection of phototoxins" (Gocke *et al.*, 2000). In addition, the Scientific Committee of Cosmetology guideline on photogenotoxicity testing does not recommend the use of an external metabolic activation system because present scientific knowledge does not allow the definition of standard conditions for testing the effect of light on a chemical in the presence of a metabolic activation system. Thus, a full irradiation dose-response curve of the test system was determined in the absence of test compound without the application of metabolic activation system in order to avoid the undesirable genotoxicity and cytotoxicity caused by direct DNA absorption. In addition, as the recommendation made by an international group to irradiate with a mixture of both UVA and UVB, the doses of UVB and UVA was determined and reported separately.

Our statistical analysis showed that there was a good correlation between photogenotoxicity test and rodent photocarcinogenicity outcome although the small number of substances analyzed reduces the power of statistical analysis. Out of 5 test substances, 4 showed a

positive outcome in photo-comet assay while 8-MOP found negative. The results suggested by others (Brendler-Schwaab *et al.*, 2004), in which 8-MOP was concluded to be negative in photo-comet assay, showed consistency with our results. Brendler-Schwaab *et al.* (2004) suggested that, due to its DNA-DNA cross-linking activity under UV-irradiation, 8-MOP led to a negative result in the photo-comet assay. In our previous results, out of 5 test compounds tested, 3 showed a positive outcome in photo-Ames assay (Hong *et al.*, 2005), suggesting that photo-Ames assay have relatively low sensitivity (the ability of a test to predict carcinogenicity). Conversely, all compounds tested were appeared to be positive in photo-CA assay (unpublished results), suggesting that the photo-CA test is most sensitive to the photogenotoxic effect of UVB-irradiation. However, the photo-CA test is particularly difficult to perform and sensitive to the effect of cytotoxicity. Also, the manual scoring of the slides under a microscope is the major limitation for an extensive use of the photo-CA test for screening purpose. Conversely, the comet assay presents the advantage of being relatively easy, reliable and fast enough to perform. This can be performed on every cell type, and thus is a particularly interesting endpoint in a strategy of *in vitro* photogenotoxicity testing. More importantly, the development of a reliable automatized system for scoring would enable the routine use of photo-comet assay in screening programs for photo-induced genotoxicity of chemical compounds.

Overall, the correlation of experimental data between photocarcinogens and photogenotoxins is quite convincing. Therefore, comet assay for photogenotoxicity in mammalian cells *in vitro* may be an easy hazard identification approach for photocarcinogens but the systems will have to be validated in further collaborative studies.

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