Short communication

Validation of a Simple *In Vitro* Comet Assay Method Using CHL Cells

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The comet assay has been widely used as a genotoxicity test *in vitro/vivo* for detecting initial DNA damage in individual cells. One of the difficulties of the assay is slide preparation, for which agarose top and bottom layers and a cell-containing middle layer are needed to immobilize the cells. To establish a practical methodology while maintaining sensitivity and reproducibility, we assessed a simple comet assay method with a hydrophilic slide glass (MAS-coat type, Matsunami glass Ind., Ltd.) instead of an agarose bottom layer. Ethyl methanesulfonate (EMS), mitomycin C (MMC), and *N*-nitroso dimethylamine (DMN) as genotoxic chemicals and triton X-100 (TRX) as a non-genotoxic chemical were used for validation of this method. Chinese hamster lung (CHL) cells were used. The results showed that EMS and DMN induced a significant increase in tail intensity. However, MMC, a known inter-strand cross-linker, did not increase tail intensity, and it was considered that this was because MMC-induced DNA-DNA crosslinks prevent separation of the DNA duplex. TRX did not increase tail intensity. These results are consistent with previous reports and demonstrate that the simple comet assay can clearly detect genotoxicity of chemicals other than interstrand cross-linkers.

Key words: *in vitro* comet assay, CHL cells

Introduction

The single cell gel electrophoresis (SCGE) assay, also known as the comet assay, was introduced by Ostling and Johanson (1) and independently modified by Singh *et al.* (2) and Olive *et al.* (3). The comet assay requires less time than genotoxicity tests with different end points, such as chromosome structural abnormalities and base sequence mutation. The assay requires only small amounts of tissue, not a large number of cells, and is applicable to a variety of eukaryotic cells and organs. It is expected to become the most useful method for detecting DNA damage. One of the difficulties of the comet assay is, however, slide preparation, for which agarose top and bottom layers and a cell-containing

middle layer are needed to immobilize the cells. The comet assay results can depend on slide quality, but slide preparation is technically difficult and time-consuming, and therefore, establishment of a practical methodology that maintains sensitivity and reproducibility is desirable. To simplify the methodology, we used a hydrophilic slide glass instead of an agarose bottom layer, and no agarose top layer. Unlike conventional slide specimens which have three agarose layers (4), only one layer was used. The cells were fluorescently stained with SYBR-Gold and scored automatically by a Comet Assay IV (Version 4.11 and 4.2, Perceptive Instruments Ltd., Suffolk, UK) image analyzer.

In this study, a simple comet assay method was validated *in vitro* with genotoxic and non-genotoxic chemicals using Chinese hamster lung (CHL) cells. Known genotoxic chemicals, ethyl methanesulfonate (EMS) as an alkylating agent, mitomycin C (MMC) as a DNA replication inhibitor (cross-linker), and *N*-nitroso dimethylamine (DMN) which requires metabolic activation to form DNA adducts, were selected. Triton X-100 (TRX), a detergent was selected as the non-genotoxic chemical.

Materials and Methods

Cell line and culture conditions: An adherent cell line, CHL, was used. The cells were maintained in Eagle's MEM medium (GIBCO, Invitrogen Corp., CA, USA) supplemented with 10 vol% heat-inactivated bovine serum (GIBCO), 200 µg/mL sodium pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cultures were incubated at 37°C, with 5% CO₂, and 100% humidity.

Test chemicals: EMS (CAS#62–50–0) and TRX

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(CAS#9002-93-1) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). MMC (CAS#50-07-7) and DMN (CAS#62-75-9) were purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. EMS was dissolved in DMSO (guaranteed grade, Wako) and the others in saline (medical use, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) for use.

Treatment conditions: The cells were treated with test chemicals for 4 or 6 h at 37°C two days after seeding onto $5 \times 10^4/5$ mL/Petri dishes (6 cm diameter). Exogenous metabolizing systems are required to induce DMN genotoxicity. Rat liver S9 fraction (Oriental Yeast Co., Ltd., Tokyo, Japan) prepared from male Sprague-Dawley rats that had received a combination of intraperitoneal injections of phenobarbital and 5,6-benzoflavone to induce drug-metabolizing enzymes was used at a concentration of 5 vol% for the treatment medium.

MAS-coat type slide glass: Hydrophilic MAS-coat type slide glasses (Cat#TF0215M) were provided by Matsunami Glass Ind., Ltd. (Osaka, Japan, http://www.matsunami-glass.co.jp/english/index_e.html). The slide glass had 2-wells and their surfaces were treated with hydrophilic silane to promote agarose adherence. Water-repellent barrier was coated outside of the wells allowing easy application of the samples. It was originally developed for frozen sections of pathological tissue specimens.

Alkaline comet assay: Immediately after treatment of cells in Petri dishes with test chemicals, the medium was poured out and the cells were washed out once with phosphate buffered saline. CHL cells were then harvest-

ed by trypsinization and sampled into a new tube. The tube was centrifuged and the supernatant was removed. The cells were washed once with 1 mL of cold HBSS (Hanks' Balanced Salt Solutions, GIBCO) containing 20 mM EDTA and 10 vol% DMSO and re-suspended in 0.5 mL of the cold HBSS. In accordance with the method shown in Table 1, specimens were prepared using a MAS-coat type slide glass instead of the conventional agarose bottom layer (1,3,4). The cells were thoroughly mixed with 0.5 w/v% low-melting agarose and an aliquot of the mixture was spotted onto a slide glass. After the gel had completely solidified, the slide specimens were immersed in chilled lysing solution overnight in a refrigerator. The slide specimens were rinsed and left in the electrophoresis solution for 20 min to allow the duplex DNA to unwind before electrophoresis. The cells were stained by SYBR-Gold (Molecular Probes, Invitrogen Corp., CA, USA) and at least 100 cells per dose were examined under a fluorescence microscope (BX50 and BX51, Olympus Corp., Tokyo, Japan) connected to the comet assay scoring system and equipped with a U-MNIBA3 filter (excitation: 470-495 nm, absorbing: 510-550 nm, Olympus Corp., Tokyo, Japan). DNA damage was quantified automatically as tail intensity (percentage of DNA migrated from the nucleus to tail, taking DNA in the nucleus as 100%).

Statistical analysis: Significant differences in tail intensity between the chemical-treated group and the solvent-treated group were evaluated by Dunnett's multiple comparison test ($P < 0.05$).

Results and Discussion

EMS: EMS was examined at 6 dose levels, 62.5,

Table 1. A basic protocol for the simple comet assay method

Item		Procedure
Cell	Cell (A)	Cells in HBSS with 20 mM EDTA and 10 vol% DMSO [#] (pH 7.5)
Gel	Bottom layer (B)	MAS-coat type slide glass
	Cell-containing middle layer (C)	0.5 w/v% low-melting agarose in PBS
	Top layer	Nothing
Specimen		Mixture of (A):(C) = 1:9 on (B)
Lysis	Solution	2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 10 vol% DMSO [#] , and 1 vol% Triton X-100 [#] in ultrapure water (pH 10)
Rinse	Condition	Overnight in refrigerator (4°C)
	Solution	Ultrapure water
	Condition	Flushing
Electrophoresis	Solution	0.3 M NaOH and 1 mM EDTA in ultrapure water (pH > 13)
	Condition	Unwinding: 20 min (< 10°C)
Neutralization		Electrophoresis: 15 min at 0.3 A (< 10°C)
Dehydration		0.4 M Tris-base in ultrapure water (pH 7.5) at least 5 min
Staining		Ethanol at least 5 min
Analysis (scoring)		SYBR-Gold for 10 min
	Software	Comet IV image analyzer
	Number	At least 100 cells per dose
	Parameter	Tail intensity (%)

HBSS: Hanks' Balanced Salt Solutions, #: Add just before use.

Fig. 1. Histogram of DNA damage induced in CHL cells after treatment with the test chemicals. CHL cells were treated with EMS (a), MMC (b), or TRX (d) for 4 h without metabolic activation, and were treated with DMN (c) for 6 h with metabolic activation. The distributions of tail intensity for 100 cells treated with EMS (a), MMC (b), or TRX (d) and for 200 cells treated with DMN (c) in each dose are shown.

125, 250, 500, 1000, and 2000 $\mu\text{g/mL}$ without metabolic activation. The results of the comet assay (Fig. 1a and 2a) show a significant dose-related increase in tail intensity at 250 $\mu\text{g/mL}$ and greater. At 2000 $\mu\text{g/mL}$, the highest dose level, the comet image could not be analyzed because all heavily damaged cells exhibited microscopic images of small or non-existent heads and large diffuse tails ("hedgehogs"). EMS is reported to be positive for the Ames assay, *in vitro* micronucleus test

using human lymphocytes, and *in vivo* unscheduled DNA synthesis (UDS) using mouse germ cells by TOXNET (Toxicology Data Network, <http://toxnet.nlm.nih.gov/cgi-bin/sis/search>). It was also demonstrated that EMS was clearly positive in the comet assay using CHO cells (5).

MMC: MMC was examined at 6 dose levels, 0.0313, 0.0625, 0.125, 0.25, 1, and 4 $\mu\text{g/mL}$ without metabolic activation. The results of the comet assay (Fig. 1b and

Fig. 2. Response of DNA damage induced in CHL cells after treatment with the test chemicals. CHL cells were treated with EMS (a), MMC (b), or TRX (d) for 4 h without metabolic activation, and were treated with DMN (c) for 6 h with metabolic activation. DNA damage was measured by the comet assay as tail intensity. Each data point in box plots represents the mean ($\pm$ SEM) of 100 cells treated with EMS (a), MMC (b), or TRX (d) and of 200 cells treated with DMN (c) for each dose. *Significant increase from solvent control by Dunnett's multiple comparison test ($P < 0.05$).

2b) show no significant increase in tail intensity up to 4 $\mu\text{g/mL}$. There were only 35 analyzable images at the highest dose of 4 $\mu\text{g/mL}$. MMC, which is a known interstrand cross-linker, did not increase tail intensity, and was considered to have induced DNA-DNA cross-links, preventing separation of the DNA duplex (5,6). MMC is reported to be positive for the Ames assay using *Escherichia coli* WP2, *in vitro* micronucleus test using human lymphocytes, *in vivo* micronucleus test, and MLA assay using L5178Y cells by TOXNET.

DMN: DMN was examined at 7 dose levels, 0.1, 10, 100, 500, 1000, 3000, and 5000 $\mu\text{g/mL}$ with metabolic activation. The results of the comet assay (Fig. 1c and 2c) show a significant dose-related increase in tail inten-

sity at 10 $\mu\text{g/mL}$ and greater. At dose levels of 500 $\mu\text{g/mL}$ and greater, the comet images could not be analyzed because only hedgehogs were observed. The assay for DMN without metabolic activation was performed concomitantly at the same dose levels. The results of the assay showed no significant increase in tail intensity at up to 5000 $\mu\text{g/mL}$ (data not shown). DMN is reported to be positive for the Ames assay with metabolic activation, *in vitro* sister-chromatid exchange (SCE) with metabolic activation using human lymphocytes, *in vivo* micronucleus test, and MLA assay with metabolic activation using L5178Y cells by TOXNET. Winter *et al.* demonstrated that DMN clearly induced tail intensity in an *in vitro* comet assay using human hepatocellular lines

expressing metabolic enzymes (7).

TRX: TRX was examined at 6 dose levels, 6.25, 12.5, 25, 50, 100, and 200 $\mu\text{g/mL}$ without metabolic activation. The results of the comet assay (Fig. 1d and 2d) show no significant increase in tail intensity at up to 100 $\mu\text{g/mL}$. At 200 $\mu\text{g/mL}$, the highest dose level, only 17 comet images were able to be analyzed and a significant increase in tail intensity was noted. This was considered due to an irrelevant positive response, because severe cytotoxicity, determined by dye-exclusion assay (trypan blue), was noted at only this dose. TRX is a detergent and an absolutely non-genotoxic agent (8).

In conclusion, the simple *in vitro* comet assay method using MAS-coat type slide glasses can clearly detect genotoxic chemicals other than DNA cross-linking agents such as MMC. Because the original comet assay also is unable to detect DNA cross-linking agents, the failure is a feature of the comet assay, and not due to the methodology (5,6,9). The sensitivity and reproducibility of the simple comet assay method must be equal to the conventional method, although it contributes to reduce labor and time. It could hold promise for the application to a high-throughput comet assay.

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