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Letter

In vitro comet assay in cultured human corneal epithelial cells

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ABSTRACT — Topical drug treatment of the eye exposes ocular tissues to a high drug concentration. Genotoxicity assessment in ocular tissues has not been established to date. Therefore, we investigated the *in vitro* comet assay by incubating cultured human corneal epithelial (HCE-T) cells with known mutagens. The alkaline comet assay was conducted to measure the DNA strand breakage yield. When the cells were incubated with methyl methanesulfonate (MMS) for 1 hr, hydrogen peroxide (H_2O_2) for 15 min and actinomycin D (AMD) for 1 hr, statistically significant increases of percentage (%) DNA in the tail were noted in MMS-, H_2O_2-, and AMD-treated cells at 100, 10, and > 10 μM, respectively. When the cells were treated with mitomycin C (MMC) or 5-bromouracil (5-BrU), %DNA in the tail was unchanged even at the highest concentration. Hedgehog cells were found in MMS- and H_2O_2-treated cells at 1000 and > 100 μM, respectively. The response to each compound was consistent with results previously reported in other cells. In conclusion, the *in vitro* comet assay using HCE-T cells can detect DNA strand breakage induced by mutagens. This method has a possibility to become a conventional screening tool to assess the genotoxicity of drugs applied to ocular surface.

Key words: *In vitro* comet assay, Cultured human corneal epithelial cells, Eye

INTRODUCTION

In general, topical drug application has the advantage of a relatively lower systemic exposure than systemic administration (*e.g*., oral, p.o., and intravenous, i.v. routes). However, drug concentrations at the administration site attain very high levels following topical application. Attempts to assess the genotoxicity at such a high exposure level using systemic methods of examination would not succeed because of the acute toxicity of the drug itself. Currently, there is no recommended method for the genotoxicity evaluation of topically applied ophthalmic drugs, although there are some references in the literature (Ye *et al*., 2011, 2012). Direct drug application into the eye using formulations such as eye-drops leads to a high drug concentration at the administration site, especially on the corneal surface (Acheampong *et al*., 2002). In addition, there exist reports regarding neoplastic transformation of corneal epithelium in human (Gichuhi *et al*., 2014) and animals (Horikiri *et al*., 1994; Ogawa *et al*., 2010). Corneal cells are considered appropriate for genotoxicity evaluation of topically applied ophthalmic drugs.

On the other hand, in the early phase of the drug development process, *in vitro* testing systems are useful because of the limited sample size of each compound required during the screening of a large number of compounds.

Therefore, we investigated the *in vitro* comet assay using cultured human corneal epithelial (HCE-T) cells, in order to establish a genotoxicity assessment method for topically applied ophthalmic drugs. For the purpose of this study, well-known mutagens including methyl methanesulfonate (MMS), actinomycin D (AMD), hydrogen peroxide (H_2O_2), mitomycin (MMC) and 5-bromouracil (5-BrU) were used as the test substances (Driessens *et al*., 2009; Henderson *et al*., 2003; Lundin *et al*., 2005; Olive and Banath, 1997; Surrallés *et al*., 1995).

MATERIALS AND METHODS

Cell culture

Simian virus (SV) 40-immortalized human corneal epithelial (HCE-T) cells were kindly provided by Dr. Kaoru Araki-Sasaki (Araki-Sasaki *et al*., 1995), and were cul-

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tured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Grand Island, NY, USA), supplemented with 5% inactivated fetal bovine serum (Gibco), 10 ng/mL human epidermal growth factor (Gibco), 5 μg/mL insulin (Wako, Osaka, Japan), and 40 μg/mL gentamicin (Gibco). The cells were cultured in cell culture flasks at 37°C in an atmosphere of 95% air and 5% CO_2. Subconfluent cultures were removed by trypsin (TrypLE™ Express, Gibco). The cells were seeded in a 12-well culture plate at a density of 1×10^5 cells/well. Then, 24 hr later, the cells were used for the comet and cytotoxicity assays.

Mutagens

The following compounds were used for the confirmation of genotoxicity against the HCT-E cells; MMS (Wako) as a DNA alkylator, AMD (Wako) as a DNA intercalator, H_2O_2, (Wako) as an oxidant, MMC (Sigma-Aldrich, St. Louis, MO, USA) as a DNA cross-linker, and 5-BrU (Alfa Aesar, Heysham, UK) as a base analogue.

Exposure to mutagens

When the cells attained approximately 80% confluence, they were washed with the serum-free medium and immediately incubated in serum-free medium containing the mutagens for 1 hr at 37°C (except H_2O_2, which was for 15 min at 4°C). Regarding with H_2O_2, the condition of incubation with cells was set for 15 min at 4°C according to the previous reports (Behravan *et al*., 2011; Kreja and Finking, 2002). All mutagens were dissolved in distilled water (Otsuka Pharmaceutical Factory, Tokushima, Japan) or dimethyl sulfoxide (DMSO, Nacalai Tesque, Kyoto, Japan), and then diluted in DMEM/F12 medium. The vehicles for each compound were used negative controls. The maximum concentration of each mutagen was decided based on their cytotoxicity or solubility to the vehicles. Specifically, the concentrations that induced moderate cytotoxicity were set as the maximum. When no toxic effect were observed, the highest concentration was set at 1000 μM or at nearly the maximum solubility of the compound. The final concentration of AMD in the culture medium was 100 μM.

Cytotoxicity assay

The cytotoxicity of the mutagens against HCE-T cells was determined using the cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan). The cells were seeded in a 12-well culture plate at a density of 1×10^5 cells/well. Then, 24 hr later, the cells were washed with serum-free medium and then incubated in serum-free medium containing varying concentrations of mutagens. After incubating for 1 hr at 37 °C or 15 min at 4°C (H_2O_2), CCK-8 was added to each well, and the cells were further incubated for 1 hr at 37°C. Then, the culture medium was transferred to a 96-well culture plate, and the absorbance was read at 450 nm using a plate reader (Multiskan JX, Thermo Fisher Scientific, Waltham, MA, USA). The relative survival was calculated as follows: absorbance of treated sample/absorbance of control.

Alkaline comet assay

The cells were seeded in a 12-well culture plate at a density of 1×10^5 cells/well. Then, 24 hr later, the cells were washed with serum-free medium and then incubated in serum-free medium containing varying concentrations of mutagens. After incubating for 1 hr at 37°C or 15 min at 4 °C (H_2O_2), the cells were washed with D-PBS (-) (Nacalai Tesque) on ice. The cells were harvested with a cell scalper (AGC Techno Glass, Tokyo, Japan) in D-PBS (-) and then combined with comet LMAgarose (Trevigen Inc., Gaithersburg, MD, USA) and placed on slides (CometSlide HT, Trevigen Inc.). The agarose was allowed to harden for 10 min at 4°C. The slides were then immersed in lysis solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, and 1% (v/v) Triton X-100 (Nacalai Tesque) overnight at 4°C. Then, they were placed in the alkaline unwinding solution (200 mM NaOH and 1 mM EDTA, pH > 13) for 20 min at room temperature. The electrophoresis was conducted in the same solution at 21 V and 300 mA for 30 min at 4°C. The slides were washed twice with MilliQ water (Millipore, MA, USA), and then the cells were fixed on the slides with ethanol for 10 min. The slides were drained, stained with the SYBR Green I staining solution (Invitrogen, Carlsbad, CA, USA), mounted using prolong gold (Invitrogen), and observed using a BX60F-3 fluorescent microscope (Olympus, Tokyo, Japan). The mean %DNA in the tail was analyzed using the Comet-Score program version 1.5.2.6 (TriTec, Stewart Road Sumerduck, VA, USA). We excluded comets that overlapped with others, were poorly stained, showed a morphological defect, or were artifacts (JEMS/MMS, 2014), and then 50 cells were selected. We counted cells with small head or no visible head or if the head and tail appear separate as "hedgehog" among 50 cells. Hedgehog cells were not scored % of DNA in the tail. The treatment of slide was masked to experimenter when analysis of hedgehog and % of DNA in tail were carried out.

Statistical analysis

Results of the %DNA in the tail were expressed as the

mean ± standard deviation (S.D.). The statistical differences ($\alpha = 0.05$, two-sided test) between the vehicle and positive controls, and vehicle and mutagens were determined using a *t*-test and Dunnett's multiple comparison test, respectively (JMP® version 11.0.0, SAS Institute Inc.). The number of hedgehog cells was counted, and no statistical analysis was applied.

RESULTS AND DISCUSSION

The survival rates of cells treated with mutagens are shown in Table 1. The survival of the H_2O_2- and MMC-treated cells decreased concentration-dependently. MMS and 5-BrU did not cause cell death even at 1000 μM which was set as the highest concentration. AMD did not induce cell death at 100 μM so that the highest dose of AMD was selected based on its solubility to the vehicles.

Typical comet images of the HCE-T cells treated with MMS are shown in Fig. 1. Cells which have small or no visible head and cells which showing separation of head with tail were judged to be the hedgehog. Typical hedgehog cells, due to its small head, are shown in Fig. 1E (the cells with white arrows).

MMS- and AMD-treated cells showed a concentration-dependent increase in the amounts of DNA fragments (Figs. 2A and 2C). Statistically significant differences were observed in the MMS- and AMD-treated cells at 100 and ≥ 10 μM, respectively. We carried out an additional comet assay using 200 μM of MMS to compare the DNA damage with that at 100 μM. The mean ± S.D. of 3 replicates at 200 μM was 45.6 ± 6.1, indicating a 2-fold higher effect than that of 100 μM. Therefore, we used 200 μM of MMS as a positive control in further experiments in accordance with certain differences from vehicle control.

As shown in Table 2, the counts of hedgehog cells increased markedly in the H_2O_2-treated cells at 100 μM. At 100 and 1000 μM of H_2O_2, the hedgehog cells were a total of 150/150 cells and a total of 149/150 cells, respectively. The increase in the %DNA in tails of cells treated with 10 μM H_2O_2 was statistically different from the control, although the mean value at this concentration was approximately 1.5-fold higher than that of vehicle control (Fig. 2B). Therefore, it appeared that the cells experienced severe DNA damage at those concentrations, suggesting that the sensitivity of HCE-T cells to H_2O_2 drastically changed between 10 and 100 μM.

In the present study, HCE-T cells showed an increase in DNA fragments following treatment with MMS (alkylator), AMD (DNA intercalator), and H_2O_2 (oxidant). These results on the comet assay for MMS, H_2O_2 and AMD are consistent with previous *in vitro* comet assays using other kind of cells such as the Chines Hamster Ovary cells (MMS and H_2O_2), PCCI3 rat thyroid cell line (H_2O_2), and SiHa spheroids (AMD) (Driessens *et al*., 2009; Olive and Banath, 1997; Sekihashi *et al*., 2003).

As mentioned above, the cytotoxicity of MMC was dose-dependent, and cell survival rates decreased to approximately 50% at 1000 μM (maximum concentration). For 5-BrU, the cell survival rate did not decrease even at the maximum concentration. We investigated DNA damage following treatment of cells with MMC and 5-BrU at 1000 μM; however, no increase in %DNA

Table 1. Cytotoxicity of the mutagens tested.

Mutagen	Vehicle	Concentration (μM)			
		Cell survival rate (% of control)			
MMS	Distilled water	1	10	100	1000
		94.3, 103.3 (98.8)	97.0, 92.4 (94.7)	93.5, 103.5 (98.5)	94.3, 93.5 (93.9)
H_2O_2	Distilled water	1	10	100	1000
		98.9, 105.6 (102.3)	98.6, 103.2 (100.9)	83.8, 87.0 (85.4)	73.6, 74.3 (74.0)
AMD	DMSO	0.1	1	10	100
		96.7, 90.1 (93.4)	94.9, 90.8 (92.9)	94.6, 95.3 (94.9)	90.4, 91.8 (91.1)
MMC	Serum free medium	1	10	100	1000
		97.8, 105.8 (101.8)	89.3, 85.8 (87.6)	66.2, 68.0 (67.1)	50.7, 49.3 (50.0)
5-BrU	DMSO	1	10	100	1000
		101.2, 104.5 (102.8)	99.6, 98.4 (99.0)	98.0, 101.6 (99.8)	101.2, 100.4 (100.8)

Individual data are shown and the mean of them are shown in parenthesis. We selected the following concentrations as doses for alkali comet assay: 1, 10, 100, and 1000 μM for MMS, H_2O_2, MMC, and 5-BrU as well as 0.1, 1, 10, and 100 μM for AMD. Control means the vehicle-treatment, and is expressed as 100%.

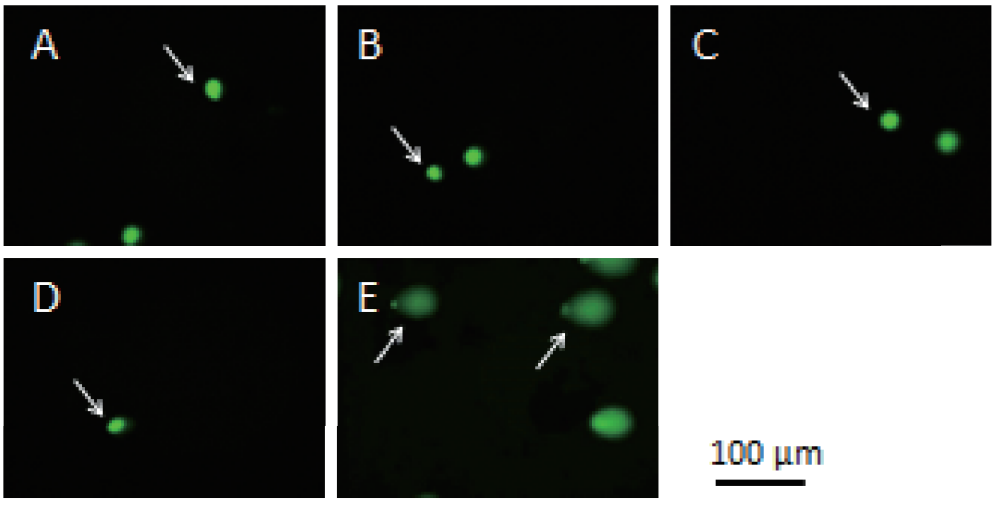

Fig. 1. Typical comet images of human corneal epithelial (HCE-T) cells treated with methyl methanesulfonate (MMS) or vehicle. Cells were treated with MMS or vehicle for 1 hr with (A) vehicle (distilled water), (B) 1 µM MMS, (C) 10 µM MMS, (D) 100 µM MMS, and (E) 1000 µM MMS. The %DNA in the tail was measured using focused cells in photographs. Intensity of cell brightness (arrows) was (A) 7.3, (B) 5.1, (C), 5.7, (D) 13.3, (E) 75.0 (left) and 74.0% (right). Comets in photograph (E) were determined to be hedgehog because small head were noted.

Table 2. Counts of hedgehog cells.

Mutagen	Concentration (µM)					
	Number of hedgehog cells (hedgehog cells/ 50 comets)					
MMS	Vehicle	1	10	100	1000	PC*
	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	50, 50, 50	10, 1, 0
H_2O_2	Vehicle	1	10	100	1000	PC
	0, 0, 0	0, 0, 0	0, 0, 0	50, 50, 50	50, 49, 50	0, 1, 0
AMD	Vehicle	0.1	1	10	100	PC
	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 1
MMC	Vehicle	1	10	100	1000	PC
	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
5-BrU	Vehicle	1	10	100	1000	PC
	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0

Cells showing small or no head were counted in 50 comets on each slide. All counts from 3 experiments are presented in the lower lines. *MMS at 200 µM was used as a positive control (PC).

of the tail was observed with these compounds at that dose (Figs. 2D and 2E). The results of the MMC-treated cells were consistent with those reported in other kinds of cells such as L5178Y (Miyamae *et al*., 1998). The comet assay is known not to detect DNA damage induced by a cross-linker after a 3-h exposure (Miyamae *et al*., 1998) because DNA cross-linkers do not induce single or double strand breaks in DNA. Base analogues induce mutation of DNA but do not break it. Therefore, the comet assay would require further modifications to enable the detection of DNA damage by DNA cross-linkers (Miyamae *et al*., 1997) and base analogues.

During the comet assay, the exposure period to mutagens was set to 1 hr (15 min for H_2O_2). Drugs that are

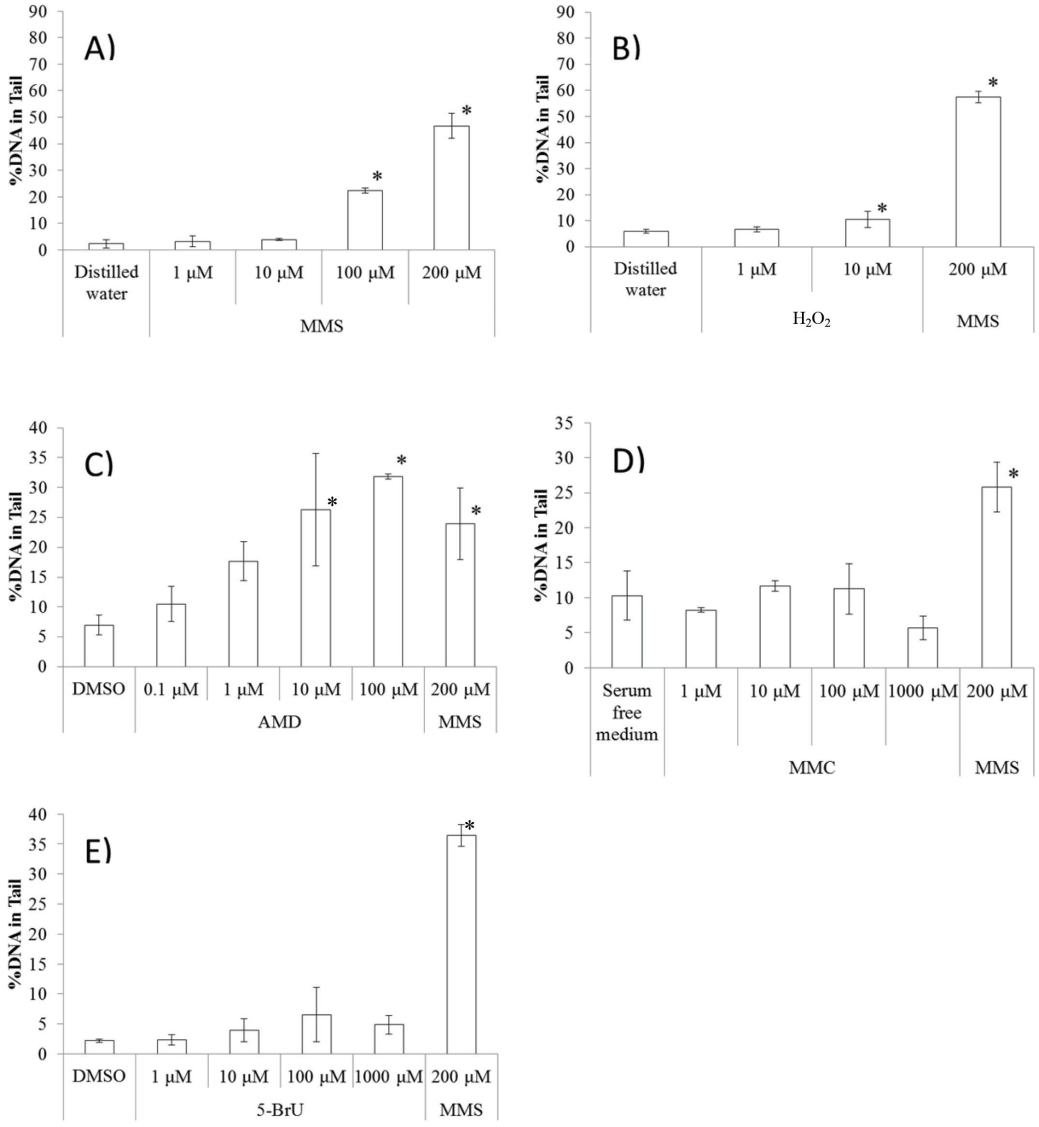

Fig. 2. Level of DNA damage in human corneal epithelial (HCE-T) cells treated with mutagens and vehicle control measured using comet assay. Each bar shows mean with S.D. of intensity of % DNA in tail from 3 independent experiments. Fifty comets were measured in each assay. Each figure shows results of (A) MMS, (B) H_2O_2, (C) AMD, (D) MMC, and (E) 5-BrU. Measurement of %DNA in tail were not performed because all or almost cells were judged the hedgehog cells in MMS at 1000 μM and in H_2O_2 at 100 and 1000 μM (see Table 2). Dunnett's multiple comparison test was performed to determine statistical analysis ($^*p < 0.05$, two-sided). No significant difference was detected for treatments with MMC and 5-BrU. MMS, methylmethanesulfonate; H_2O_2, hydrogen peroxide; AMD, actinomycin; MMC, mitomycin; 5-BrU, 5-bromouracil.

administered to the eye are rapidly eliminated by dilution with tear fluid and drainage via the nasolacrimal duct (Mishima *et al*., 1966; Regnier, 2013). Therefore, long drug exposure periods would not be relevant for the assessment of eye drop drugs. In the present study, the mutagens tested yielded DNA strand breakage similar to the *in vitro* comet assay performed using other cells (Sekihashi *et al*., 2003), suggesting that the shorter exposure period is suitable for the assessment of topically applied drugs.

In the present study, we did not perform evaluations under metabolically activated condition (*i.e*., in the presence of S9 mixture). The ocular surface is known to have a low level of drug metabolizing enzymes, although some

cytochrome P450 (CYP) enzymes have been detected in ocular tissues (Zhang *et al*., 2008). In addition, Kölln and Reichl (2012) reported that the HCE-T cells have the same drug metabolizing enzymes as the human corneal epithelium. These descriptions imply that metabolizing systems is not essential for evaluating drugs applied to the ocular surface using the HCE-T cells. However, further studies are necessary regarding significance of the metabolizing systems in a comet assay using cultured corneal epithelial cells.

The sensitivity of the present comet assay using the HCE-T cells is almost equivalent to those of the previous comet assay systems using the human lymphocytes (Speit *et al*., 2012) and the human TK-6 cells (Azqueta *et al.*, 2013). The present method has a characteristic to use the ocular cells of the corneal epithelium. Our present results indicate a possibility to become a conventional screening tool to assess the genotoxicity of drugs applied to ocular surface.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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