Effect of in vitro exposure time on comet assay results

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Summary

Some mutagens are inactivated rapidly by components included in culture media, especially by serum. Long incubation periods may not be appropriate for the comet assay because DNA lesions may be repaired during the time that mutagens are inactivated, leading to false negative results. We questioned how the exposure period of Chinese hamster ovary cells to 8 unstable mutagens affected outcome of the assay.

Although the longest biological half-life of the test mutagens was 1.98 h, four were positive following 0.5–24 h incubations while other four were positive only when the incubation period was ≤ 4 h, suggesting that the DNA damage was repaired and the mutagens were inactivated. The rapid inactivation of mutagens in the medium did not affect whether the outcome of the comet assay was positive or negative when cells were exposed for 1–4 h. Based on these results, we concluded that long exposure should not be employed for the compounds that are unstable in culture media, and appropriate incubation time should be determined for them individually.

Keywords: in vitro comet assay, exposure period, inactivation, biological half-life

Introduction

Because exposure period is an important factor affecting the outcomes of in vitro genotoxicity test, extensive studies to determine choice appropriate exposure periods in the TK mutation (Moore et al., 2000) and chromosome aberration tests (Galloway, 2000) have been conducted. The alkaline (pH > 13) comet assay introduced by Singh et al. (1988) is a rapid and sensitive procedure for quantitating DNA lesions in mammalian cells. It detects not only single strand breaks but also alkali-labile sites, DNA crosslinking, and incomplete excision repair

sites (Singh et al., 1988; Fairbairn et al., 1995). Now, the comet assay is used widely to detect chemical genotoxicity in vitro and in vivo, this assay was discussed in the 1999 International Workshop on Genotoxicity Test Procedures Workgroup held in Washington, D.C., in which consensus was reached on many important aspects of the comet assay protocol. To detect chemical genotoxicity in vitro, an appropriate exposure period was considered to be 3–6 h in the presence or absence of a metabolic activation system (Tice et al., 2000).

Some mutagens are inactivated rapidly by components included in culture media especially by serum (Jensen et al., 1977). When assessing a chemical that is unstable in culture media with the comet assay, a long exposure period would not be appropriate because the initial DNA lesions would be repaired and new ones would not be formed after inactivation of chemical, leading to false negative results. In this study, therefore, we questioned whether the exposure period to unstable chemicals affects the outcome of the assay.

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Materials and Methods

Chemicals, cells, and medium

N-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG: CAS 70-25-7), *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine (ENNG: CAS 63885-23-4), methyl methanesulfonate (MMS: CAS 66-27-3), and ethyl methanesulfonate (EMS: CAS 62-50-0) were purchased from Sigma Chemicals Inc., St. Louis, MO (U.S.A.). Methyl nitrosourea (MNU: CAS 684-93-5) and ethyl nitrosourea (ENU: CAS 759-73-9) were purchased from Nacalai Tesque, Inc., Kyoto (Japan). 4-Nitroquinoline-1-oxide (4NQO: CAS 56-57-5) and hydrogen peroxide (H_2O_2 : CAS 7722-84-1) were purchased from Wako Pure Chemical Industries, Ltd., Osaka (Japan). The chemicals were dissolved in Hanks' balanced salt solution (pH 7.0) immediately before use. Regular (GP-42) and low melting point (LGT) agarose were purchased from Nacalai Tesque, Inc., and diluted to 0.75% in physiological saline.

Chinese hamster ovary (CHO K1) cells, obtained originally from American Type Culture Collection and cloned, were grown in Ham's F12 medium (Nissui Pharmaceutical Inc., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Armour Pharmaceutical Company, Kentakee, U.S.A., Lot No. D84812) at 37 °C with 5% CO_2 .

Treatment

To study the effects of exposure periods, we treated logarithmic growth phase CHO cells (1×10^6 cells/mL treatment medium) with each chemical at 37 °C for 30 min–24 h in fresh culture medium containing FBS. Cells were sampled immediately after treatment as described below, and the percentage of viable cells was measured by the trypan blue exclusion test. The highest treatment concentration was chosen as the concentration at which cell viability after 24 h treatment was not lower than 70%.

To detect the biological half-life of each mutagen in the culture media, we incubated them at 37 °C for 0–4 hours in Ham's F12 medium containing 10% FBS. The concentration was the highest concentration used for the study of the effects of exposure periods. Cells were exposed for 1 h to the medium in which each mutagen had been incubated and were sampled immediately after exposure, as described below.

Comet assay

Seventy five μL of 1% agarose GP-42 (Nacalai Tesque, Inc., Kyoto, Japan) was quickly layered on a slide (Matsunami Glass Ind., Ltd., Osaka, Japan) and covered with another slide glass. Treated cells were suspended in 1% agarose-LGT (Nacalai Tesque, Inc.) and 75 μL of cell suspension (5×10^5 cells/75 μL) was quickly layered at the same manner after removing the slide glass.

Finally, 75 μL of 1% agarose GP-42 was quickly laid on again. The slide were lysed immediately in a solution (pH 10) of 2.5 M NaCl, 100 mM Na_2EDTA , 10 mM Trizma, 1% sarkosyl, 10% DMSO, and 1% Triton X-100 at 4 °C for 60 min (Singh et al., 1988).

The slides were placed on a horizontal gel electrophoresis platform, and covered with an alkaline solution made up of 300 mM NaOH and 1 mM Na_2EDTA (pH > 13). The slides were left in the solution for 20 min to allow the unwinding of the DNA and expression of alkali-labile sites. The power supply was set at 25 V and 250 mA. The DNA was electrophoresed for 20 min and the slides were rinsed with 400 mM Trizma (pH 7.5) to neutralize the excess alkali. Each slide was stained with 50 μL of 20 $\mu\text{g}/\text{mL}$ ethidium bromide (Wako Pure Chemical Industries, Ltd.) and covered with a coverslip.

Cells on one slide were photographed (black and white 400 ASA Fuji film) as seen with the aid of a fluorescence microscope (Olympus at 200 $\times$ magnification) equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. The length of the comet (migration) was measured for fifty cells per treatment group. The effect of chemical treatment on migration was analyzed using ANOVA and the Dunnett test.

To calculate biological half-life, the relative induced migration was obtained as the percentage of net migration value after each incubation period for that after 0 h-incubation. The approximation polynomial of relative induced migrations was obtained and the biological half-life was calculated by solving the equation obtained from the approximation polynomial.

Results

Fig. 1 shows the exposure period-response curves for CHO cells exposed to each mutagen. With any treatment condition, relative cell viability was over 70% (data not shown). Fig. 2 shows the results of exposure for 1 h to culture medium in which mutagen had been incubated for 0–4 h. Each symbol shows relative induced migration value. Broken curves are approximation curves that were calculated from approximation polynomials based on relative induced migration values.

ENU exposure yielded DNA damage after 0.5–1 h at 50–400 $\mu\text{g}/\text{mL}$. DNA migration returned to control levels after exposure for 24 h. The biological half-life of ENU in the culture medium was calculated to be 1.01 h.

MNU exposure yielded DNA damage after 0.5–24 h at 25–100 $\mu\text{g}/\text{mL}$, with the damage peaking at 2 h. The biological half-life of MNU in the culture medium was 1.07 h.

DNA damage induced by EMS at 50–200 $\mu\text{g}/\text{mL}$ peaked at 2–4 h. Exposure to the highest concentration for EMS exposure for 1 h led to a positive response. The biological half-life of EMS in culture medium was calculated to be 1.98 h.

Fig. 1 Comet assay of DNA taken from CHO cells exposed for various time periods to 8 mutagens. CHO cells were treated for 0.5–24 h with ENU at 50 ($\triangle$), 100 ($\blacktriangle$), 200 ($\circ$), or 400 $\mu\text{g/mL}$ ($\bullet$); MNU at 12.5 ($\triangle$), 25 ($\blacktriangle$), 50 ($\circ$), or 100 $\mu\text{g/mL}$ ($\bullet$); EMS at 25 ($\triangle$), 50 ($\blacktriangle$), 100 ($\circ$), or 200 $\mu\text{g/mL}$ ($\bullet$); MMS at 5 ($\triangle$), 10 ($\blacktriangle$), 20 ($\circ$), or 40 $\mu\text{g/mL}$ ($\bullet$); ENNG 2.5 ($\triangle$), 5 ($\blacktriangle$), 10 ($\circ$), or 20 $\mu\text{g/mL}$ ($\bullet$); MNNG at 0.31 ($\triangle$), 0.63 ($\blacktriangle$), 1.25 ($\circ$), or 2.5 $\mu\text{g/mL}$ ($\bullet$); 4NQO at 0.048 ($\blacktriangle$), 0.095 ($\circ$), or 0.19 $\mu\text{g/mL}$ ($\bullet$); H_2O_2 at 0.75 ($\triangle$), 1.5 ($\blacktriangle$), 3 ($\circ$), or 6 $\mu\text{g/mL}$ ($\bullet$). The symbol $\square$ indicates control. Significant difference: * $p < 0.05$.

Fig. 2 Comet assay of DNA taken from CHO cells exposed to 8 mutagens that were preincubated in culture medium containing FBS for 0–4 h. CHO cells were exposed for 1 h to culture medium in which mutagens at the concentrations shown in the figures had been incubated for 0–4 h. Each symbol shows relative induced migration value. Curves shown as broken lines are approximation polynomials calculated based on relative induced migration values.

Exposure to high concentrations of MMS for 0.5–24 h led to positive responses while exposure to the low concentration did not, even after 24 h. The biological half-life of MMS in the culture medium was calculated to be 1.33 h.

ENNG at 2.5–20 $\mu\text{g/mL}$ led to positive responses. DNA damage induced by ENNG at 20 $\mu\text{g/mL}$ peaked following exposure for 2 h and then decreased. Its biological half-life of ENNG in the culture medium was calculated to be 1.11 h.

DNA damage induced by MNNG peaked at 4 h at 0.31–2.5 $\mu\text{g/mL}$. DNA damage was not evident following expo-

sure for 24 h at 0.31 $\mu\text{g/mL}$. The biological half-life of MNNG in the culture medium was calculated to be 0.96 h.

DNA damage induced by 4NQO at 0.048–0.75 $\mu\text{g/mL}$ peaked following exposure for 1 h and then decreased. The biological half-life of 4NQO in the culture medium was calculated to be 1.74 h.

CHO cell exposure to H_2O_2 for 30 min at 3 and 6 $\mu\text{g/mL}$ led to positive responses. DNA damage had decreased with exposure time starting at 1 h or more. On the other hand, CHO cell exposure to H_2O_2 at 0.75 and 1.5 $\mu\text{g/mL}$ did not lead to positive responses. The biological half-life of H_2O_2 in the culture medium was calculated to be 0.65 h.

Discussion

Among the studied mutagens, EMS showed the longest biological half-life, 1.98 h. Thus, the mutagens were inactivated rapidly in the medium, which contained FBS. The relationship of comet assay results to treatment period was divided into two. DNA damage induced by ENU, EMS, 4NQO, and H₂O₂ decreased with exposure period and returned to control level following 24 h treatment. Considering that their biological half-lives of the mutagens were less than 2 h, it is likely that the induced DNA damage was repaired between mutagen inactivation and the sampling at 24 h. Although H₂O₂ produces oxidative DNA-strand breaks, most are repaired within 15 min (McKelvey-Martin et al., 1993), but in the present study H₂O₂ was positive after 0.5–4 h exposure. That coincides with the finding by Visvardis et al. (1997) in lymphocytes, in which DNA damage persist for over 2 h following incubation with H₂O₂. On the other hand, DNA damage induced by MNU, ENNG, MNNG, and MMS did not decrease greatly and was detected following 24 h of treatment. Thus, the DNA damage they induced tended to be difficult to be repaired.

Jensen et al. (1977) reported that the biological half-lives of 1 mg/mL MNU and 3 µg/mL 4NQO were, respectively, 8 min and 67 h in culture medium containing 15% FBS. Which components of media inactivate mutagens is not known and presumably, the biological half-lives of mutagens would differ in different media. In spite of such differences the fact that mutagens are inactivated in media is important to the choice of treatment period in the comet assay. Our results suggest that rapid inactivation of mutagens does not affect whether the outcome of the assay is positive or negative when cells are exposed for up to 4 h. Despite this, even after exposing human hepatoma HepG2 cells for 96 h to four known promutagens (cyclophosphamide, benzo[*a*]pyrene, dimethylbenz[*a*]anthracene, and 2-acetylaminofluorene), Yusuf et al. (2000) noted positive effects. It should be also taken into consideration that repair processes could be damaged by compounds that suppress DNA repair and that cells could experience

growth cessation or cell cycle arrest after exposure to DNA damaging agents (Smith and Fornace, 1996). However, long exposure periods should not be employed for compounds that are unstable in culture media, and appropriate incubation times should be determined for them individually.

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