

Regular article

Detection of DNA Single Strand Breaks Induced by Chemical Mutagens Using the Acellular Comet Assay

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In the acellular comet assay, slides with gels prepared from untreated cells are exposed after lysis to the test agents and then processed according to the standard comet assay protocol. The sensitivity of acellular comet assay was compared with that of standard assay using human lymphoblastoid WTK1 cells. Selected model mutagens were *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), methyl nitrosourea (MNU), ethyl nitrosourea (ENU), methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), bleomycin (BLM), and UVC. In the acellular assay, lysed slides were exposed to model chemical mutagens for 2 h or irradiated to UVC, and then electrophoresed immediately after chemical mutagen-treatment or 2 h after UVC-irradiation. The slides for standard assay were prepared immediately after 2 h-exposure to each chemical mutagen or 2 h after UVC-irradiation. In both assays, slides were electrophoresed at pH > 13 or pH 12 for 20 min after 20 min unwinding. BLM was positive at pH > 13 and pH 12 in both assays. UVC was positive in the standard assay but not in the acellular assay. In spite of positive responses of alkylating agents in the acellular assay at pH > 13 and pH 12, they were positive at pH > 13 but not at pH 12 in the standard assay. The positive responses in the acellular assay were greater than those in the standard assay. Our present results suggest that acellular comet assay can detect DNA single strand breaks (SSBs) as initial lesions but not alkali-labile sites generated from DNA lesion such as alkylated bases and that the sensitivity to detect SSBs as initial lesions is lower in the standard than in the acellular assay.

Key words: acellular comet assay, standard comet assay, initial DNA lesions, chemical mutagens, DNA strand breaks

Introduction

The comet assay is a rapid and sensitive procedure for quantitating DNA lesions in mammalian cells (1,2). In

this assay, cells are embedded in agarose, lysed, and subjected to an electric current in an alkaline solution. Relaxed and broken DNA fragments stream further from the nucleus than intact DNA, so the extent of DNA damage can be measured by the length of the stream. When cells are treated with base adduct-inducers or alkylating agents, the agents produce base adducts or alkylated bases that develop into DNA single strand breaks (SSBs) and/or alkali-labile sites through repairing events (3,4). Thus, although the comet assay is generally understood as a method to detect DNA initial lesions, it should be mentioned that this assay cannot detect directly initial DNA lesions, such as base adducts or alkylated bases but that this assay can detect SSBs as initial lesions and SSBs developed from alkali-labile sites under alkaline condition (pH > 12.6) (5). In the present study, initial DNA lesions are defined as DNA lesions, such as SSBs, alkylated base, base adducts, and pyrimidine dimers that produced directly by the reaction between mutagens and DNA. SSBs that generated during repairing process of initial lesions such as alkylated base, base adducts, and pyrimidine dimers are considered to be secondary lesions. Furthermore, it has been demonstrated that topoisomerase II inhibitors interfere with the breakage/reunion reaction of topoisomerase II, resulting in comet assay-detectable SSBs (6). On the contrary, it is considered that comet assay-detectable SSBs disappear through repairing events. Therefore, to understand adequately what we can detect by this assay, it is important to know how initial DNA lesions and repairing events contribute to the sensitivity of this assay.

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The acellular comet assay is one modified version of the comet assay, in which slides with gels prepared from untreated cells are exposed after lysis to test agents and then processed according to the standard comet assay protocol (7). In the acellular comet assay, since lysed cells are exposed to test compounds, any biological events do not act to affect the formation and/or disappearance of SSBs (7). In the present study, we questioned how initial DNA lesions and repairing events contribute to the sensitivity of this assay by comparing the results of standard and acellular comet assay. We selected the following model mutagens: the SN-1 type alkylating agents *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), methyl nitrosourea (MNU), and ethyl nitrosourea (ENU), the SN-2 type alkylating agents methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS), the X-ray-mimetic mutagen bleomycin (BLM), and UVC.

Materials and Methods

Chemicals: MNNG, MMS, and EMS were obtained from Sigma Chemicals Inc., St. Louis, MO (U.S.A.). MNU and ENU were purchased from Nacalai Tesque, Inc., Kyoto (Japan). They were dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Ltd.). BLM (Wako Pure Chemical Industries, Ltd.) was dissolved in physiological saline. Regular (GP-42) and low melting point (LGT) agarose were obtained from Nacalai Tesque, Inc., and were diluted to 1 % in physiological saline.

Cells: *TK*^{+/-} heterozygote of the WTK1 human lymphoblastoid cell line (kindly provided by Dr. Honma, National Institute of Health Sciences) was used. Cells were maintained in logarithmic growth using RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% horse serum (SAFC Biosciences), 200 μ g/mL sodium pyruvate, and 200 μ g/mL streptomycin at 37°C under a 5% CO₂ atmosphere.

Standard comet assay: For treatment, cells were centrifuged and re-suspended in culture medium at a concentration of 5×10^5 cells/mL, and 1 mL of cell suspension, containing each chemical mutagen was incubated for 2 h. Exposed cells were sampled immediately after chemical treatment and the percentage of viable cells was measured by the trypan blue exclusion test. For UVC-irradiation, 1 mL of cell suspension in saline (5×10^5 cells/mL) in 6 cm-dish was irradiated to a germicide lamp (National GL15, 15W, Matsushita Electric Industrial Co., Japan) as the UVC source from a distance of 15 cm at a dose of 5–30 J/m². Irradiated cells were sampled 2 h after UVC-irradiation. Treated cells were suspended in 1% agarose-LGT at 5×10^5 cells/75 μ L and 75 μ L of cell suspension was quickly layered on a fully frosted slide (Matsunami Glass Ind., Ltd., Osaka,

Japan) that was coated with 1% agarose GP-42 and covered with another slide glass. The slides were placed to allow the agarose to gel. The slides were lysed immediately at 4°C in a lysing solution (pH 10) of 2.5 M NaCl, 100 mM EDTA disodium (Na₂EDTA), 10 mM Trizma, 1% sarkosyl, 10% DMSO, and 1% Triton X-100 for one night (1). The slides were placed on a horizontal gel electrophoresis platform, and covered with pH > 13 alkaline solution made up of 300 mM NaOH and 1 mM Na₂EDTA or pH 12 alkaline solution made up of 10 mM NaOH, 300 mM NaCl and 1 mM Na₂EDTA. 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 μ L of 20 μ g/mL ethidium bromide (Wako Pure Chemical Industries, Ltd.) and covered with a coverslip. Fifty cells on one slide per dose (one slide was prepared for each dose) were examined and photographed (black and white ASA 400 Fuji film) at 200 X magnification using a fluorescence microscope (Olympus) equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. The whole length of the comet (“migration”) was measured using a scale. The effect of chemical treatment on migration was analyzed using ANOVA and the Dunnett test.

Acellular comet assay: Comet slides from untreated WTK1 cells were prepared as outlined above and the slides were lysed immediately in a lysing solution at 4°C for one night as described above. Lysed slides were neutralized in 400 mM Tris HCl buffer (pH 7.5) for 15 min and exposed to RPMI 1640 medium with 10% horse serum containing different concentrations of the test agents for 2 h at 37°C in the dark. After the treatment period, the slides were rinsed by immersion in cold distilled water, and then the slides were electrophoresed and analyzed as described above. For UVC-irradiation, neutralized slides were irradiated to germicide lamp (National GL15, 15W, Matsushita Electric Industrial Co., Japan) from a distance of 15 cm at a dose of 5–30 J/m². Irradiated slides were left for 2 h in 400 mM Tris HCl buffer immediately after UVC-irradiation, and then the slides were electrophoresed.

Results

Cell viability was $\geq 70\%$ (data not shown), showing that observed DNA migration was not due to cell death.

BLM: At pH 12 as well as at pH > 13, BLM increased migration significantly in both standard and acellular comet assays (Fig. 1a). While statistically significant increase in tail length was observed at ≥ 0.0625 μ g/mL BLM in the acellular assay, tail length increased significantly at ≥ 2.5 μ g/mL BLM in the standard as-

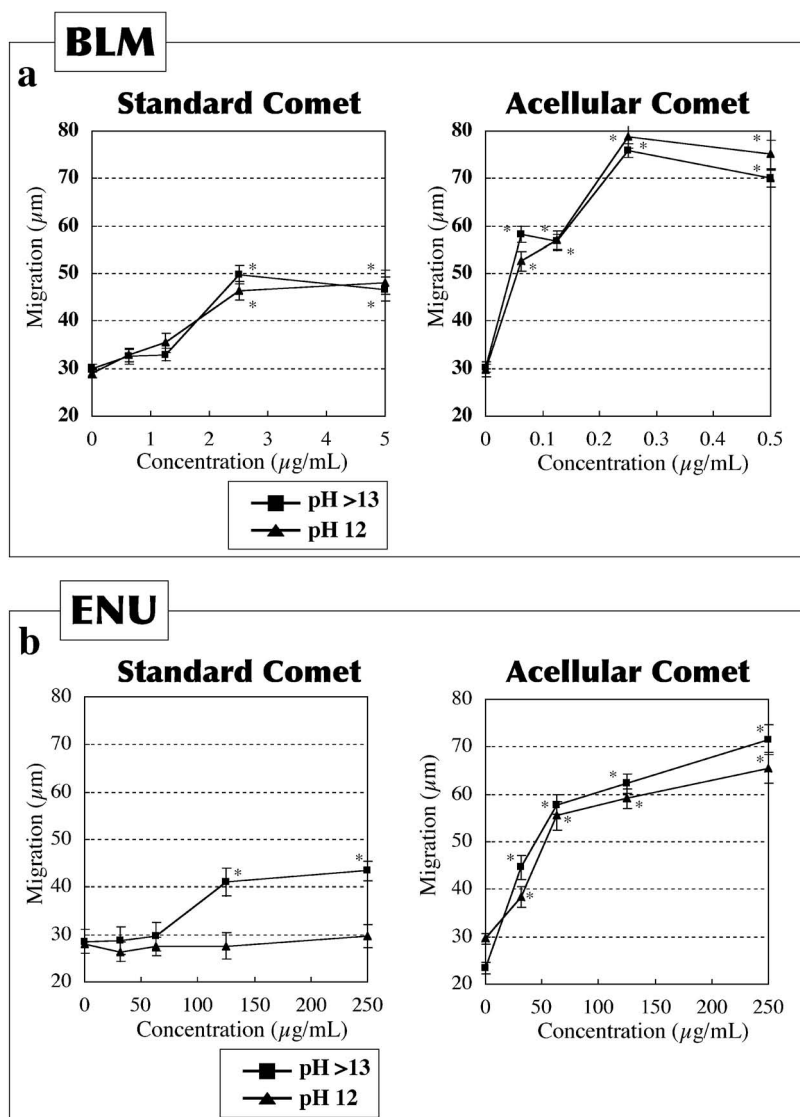


Fig. 1. Standard vs. acellular comet assay with mutagens. In the standard assay, WTK1 cells were exposed to each chemical mutagen for 2 h, or irradiated to UVC, and then comet slides were prepared immediately after the exposure, or 2 h after the irradiation. In the acellular assay, comet slides prepared using untreated WTK1 cells were exposed to each chemical mutagen for 2 h. Electrophoresis was conducted at pH 12 and pH > 13. Reproducibility was ascertained by three independent experiments and representative data are shown in this Figure. To show data for the lowest positive concentration, data for $>0.5 \mu\text{g/mL}$ BLM were omitted in the acellular assay (Fig. 1a). The error bars indicate standard errors of the mean. a, BLM; b, ENU; c, MNU; d, MNNG; e, MMS; f, EMS; g, UVC. *Significant difference from untreated control: $p < 0.05$.

say. Furthermore, tail length was greater in the acellular assay than in the standard assay.

SN-1 type alkylating agents: ENU at $\geq 125 \mu\text{g/mL}$ increased tail length significantly in the standard assay at pH > 13 but not pH 12 (Fig. 1b). In the acellular assay at pH > 13 and pH 12, ENU at $\geq 31.25 \mu\text{g/mL}$ increased tail length significantly, i.e. the lowest concentration where positive response was observed was 1/4-fold lower in the acellular than in the standard assay. MNU at $\geq 25 \mu\text{g/mL}$ increased tail length significantly in the standard assay at pH > 13 but not pH 12 (Fig. 1c). In the acellular assay at pH > 13 and pH 12, MNU at \geq

$12.5 \mu\text{g/mL}$ increased tail length significantly, i.e. the lowest concentration where positive response was observed was 1/2-fold lower in the acellular than in the standard assay.

MNNG at $\geq 0.1 \mu\text{g/mL}$ increased tail length significantly in the standard assay at pH > 13 but not pH 12 (Fig. 1d). In the acellular assay at pH > 13 and pH 12, MNNG did not lead to positive responses at studied concentration range.

SN-2 type alkylating agents: MMS at $40 \mu\text{g/mL}$ increased tail length significantly in the standard assay at pH > 13 but not pH 12 (Fig. 1e). In the acellular assay

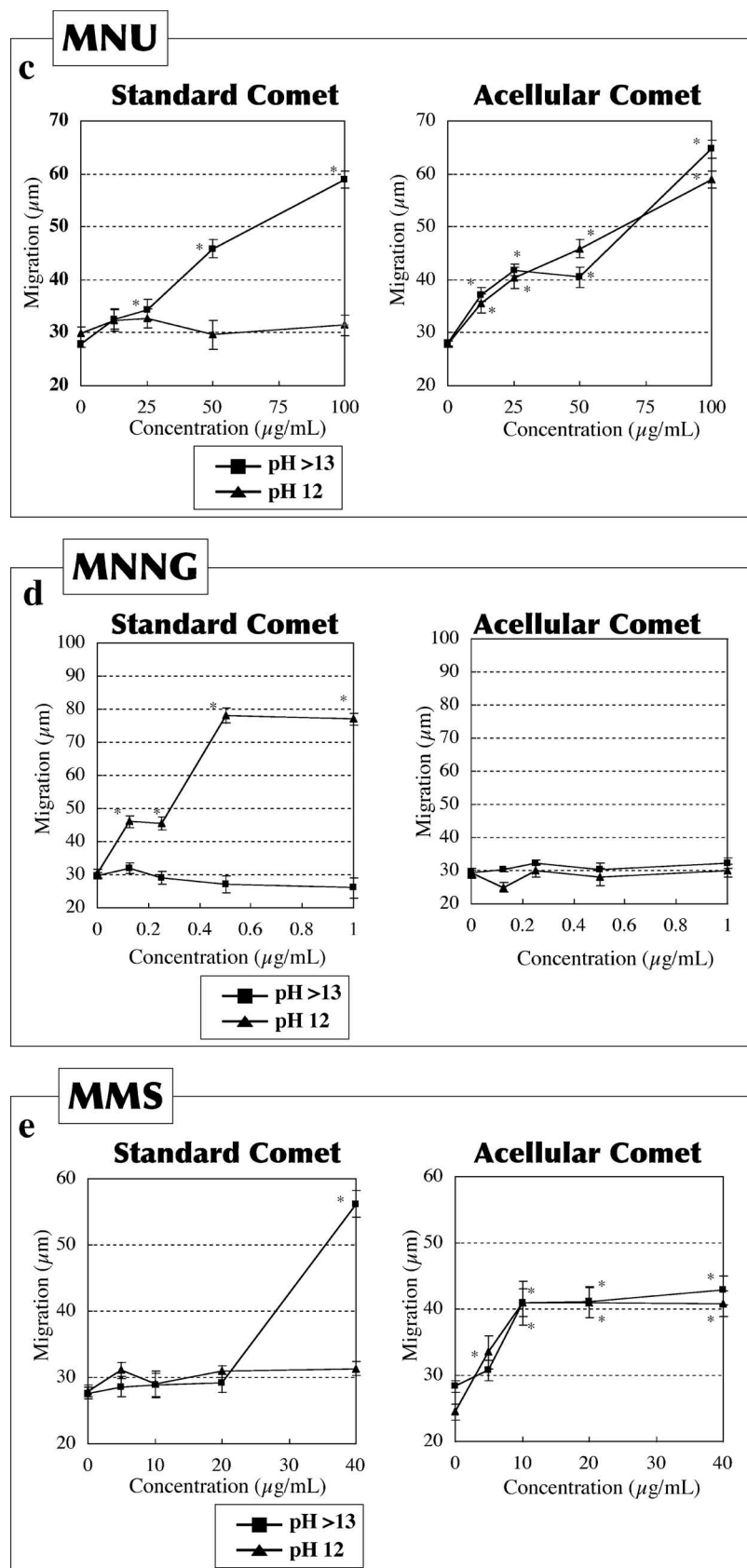


Fig. 1.

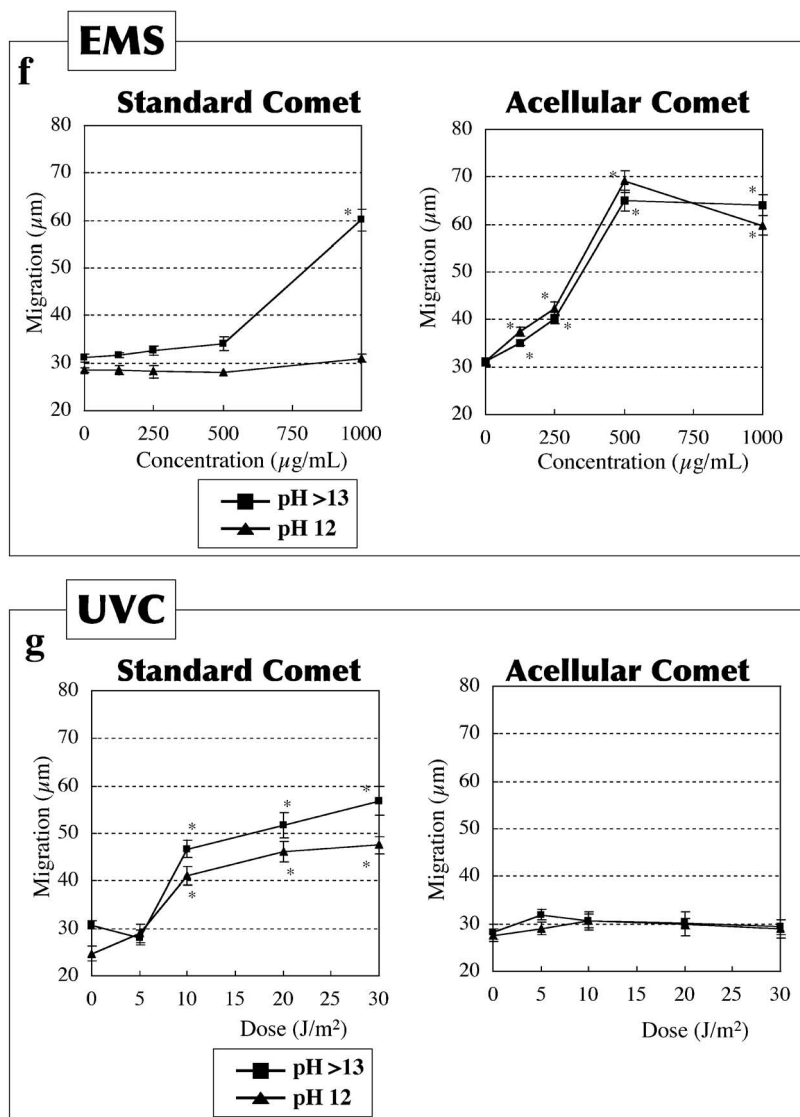


Fig. 1.

at pH >13 and pH 12, MMS at $\geq 5 \mu\text{g/mL}$ increased tail length significantly, i.e. the lowest concentration where positive response was observed was 1/8-fold lower in the acellular than in the standard assay. EMS at $1000 \mu\text{g/mL}$ increased tail length significantly in the standard assay at pH >13 but not pH 12 (Fig. 1f). In the acellular assay at pH >13 and pH 12, EMS at $\geq 125 \mu\text{g/mL}$ increased tail length significantly, i.e. the lowest concentration where positive response was observed was 1/8-fold lower in the acellular than in the standard assay.

UVC: UVC at $\geq 10 \text{ J/m}^2$ increased tail length significantly in the standard assay at pH >13 and pH 12 (Fig. 1g). In the acellular assay at pH >13 and pH 12, UVC did not lead to positive responses at studied dose range.

Exposure period effect in the acellular assay: In

the study described above, chemical exposure period of mutagen was 2 h. In the following study, to know whether the 2 h-exposure in the acellular assay is suitable, acellular assay where slides were exposed to 3 selected mutagens (BLM, MNU, and MNNG) for 15 min–24 h was conducted at pH >13 (Fig. 2). Of 3 selected mutagens, BLM is an SSB-inducer, MNU is a methylating agent that led to positive responses in both assays by 2 h-exposure, and MNNG is a methylating agent that did not lead to positive responses in the acellular assay by 2 h-exposure. The exposure to $0.5 \mu\text{g/mL}$ BLM and $100 \mu\text{g/mL}$ MNU for $\geq 30 \text{ min}$ and to $1 \mu\text{g/mL}$ MNNG for $\geq 4 \text{ h}$ increased tail length significantly (Fig. 2).

Discussion

The results obtained in this study are summarized as follows: 1) pH effects were not observed in the acellular

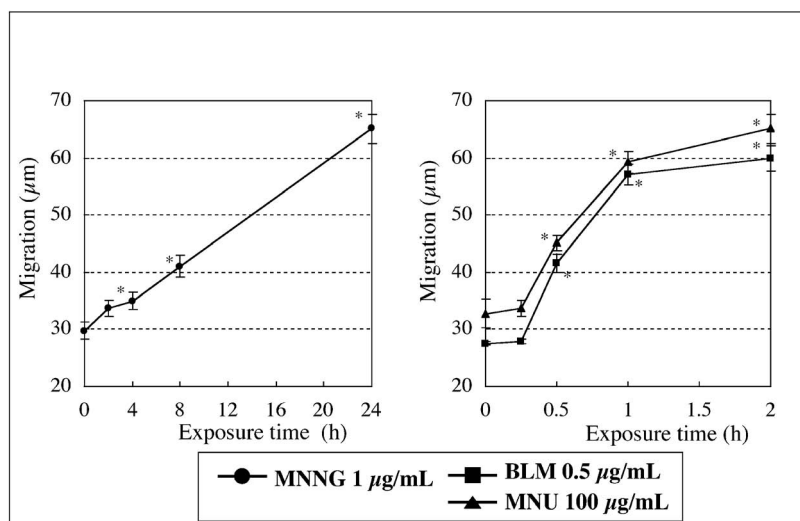


Fig. 2. DNA migration by MNU, BLM, and MNNG in the acellular comet assay as a function of exposure period. Comet slides prepared using untreated WTK1 cells were exposed for ≤ 24 h to each mutagen. Reproducibility was ascertained by three independent experiments and representative data are shown in this Figure. The error bars indicate standard errors of the mean. *Significant difference from untreated control: $p < 0.05$.

assay, 2) UVC led to negative responses in the acellular assay, 3) positive responses were obtained at pH 12 for BLM and UVC but not alkylating agents in the standard assay, 4) responses in the acellular assay is usually greater than those in the standard assay at pH 13, and 5) the responses depended on exposure time in the acellular assay. Both WTK1 and TK6 cells are derived from WIL-2 cells, and TK6 and WTK1 cells have wild type p53 and a mutant p53, respectively. Although WTK1 cells are more resistant to clastogenic effects than TK6 cells (8) and are more sensitive to genotoxic effects than TK6 cells (9), it was shown that there were not any differences in comet assay-response between WTK1 and TK6 cells in our previous study (10). Therefore, although p53 mutant WTK1 cells were used in the present study, it would be possibly assumed that p53 status would not affect comet assay-responses not only in acellular condition but also in standard condition. However, it is remained for further studies how p53 status affect comet assay-responses.

Kasamatsu *et al.* (7) showed that H_2O_2 , MNU, and BLM increased DNA migration in both standard and acellular assays and that 4-nitroquinoline 1-oxide, 5-fluorouracil, and methotrexate that require cellular function to show their genotoxicity induced increased DNA migration in the standard comet assay only. In our study, tested mutagens except for UVC led to positive responses in the standard and acellular assays, which is very similar to earlier finding by Kasamatsu *et al.* (7). In Kasamatsu *et al.* (7), however, it has not been mentioned what kinds of DNA lesions were detected in the acellular assay, standard assay, and/or both assays. In this study, to know what kinds of DNA lesions can be detected well in each assay, we differentiated SSBs from

other kinds of DNA lesions by using different electrophoresis conditions. The modes of mutagen-action, responses of standard and acellular comet assays at different pH, and expected comet images are illustrated in Fig. 3. The comet assay detects DNA damages as DNA breaks that are initially induced by an agent and are generated from AP sites at pH > 12.6 (11,12). DNA strand breaks may represent direct effect of some damaging agents. In addition, they may also be intermediates generated in cellular repair process, because SSBs are formed to cut out damaged bases and replace them with sound nucleotides during the nucleotide and base excision-repair processes.

When SSBs as initial DNA lesions are not rejoined, they would be responsible for DNA migration (Fig. 3A). For SSB-inducer such as BLM, therefore, migration would be observed in the acellular condition at pH 12 and pH > 13 and rejoining of SSBs before comet-preparation would reduce positive response in the standard assay. If the level of SSBs as initial DNA lesions is high enough to persist until comet-preparation, migration would be observed also in the standard condition at pH 12 and pH > 13 . BLM is well known to induce SSBs (13), which coincides well with the positive responses by BLM in the acellular and standard assays at pH 12. For BLM, tail length was greater in the acellular than in the standard assay. Furthermore, while BLM led to positive responses at $\geq 0.0625 \mu\text{g/mL}$ in the acellular assay, it led to negative responses at $\leq 1.25 \mu\text{g/mL}$ in the standard assay. This discrepancy would be explained as follows; although rejoining of BLM-induced SSBs before comet-preparation reduces positive responses in the standard assay, rejoining of BLM-induced SSBs does not occur in the acellular assay where any cellular func-

	Initial lesions	Repairing process	Under alkali		Comet images	
A SSBs (BLM and alkylating agents)			pH 12			Standard comet
			pH >13			
			pH 12			Acellular comet
			pH >13			
			pH 12			Standard comet
			pH >13			
B Base damages (Alkylating agents)			pH 12			Standard comet
			pH >13			
			pH 12			Acellular comet
			pH >13			
			pH 12			Standard comet
			pH >13			
C Pyrimidine dimers (UVC)			pH 12			Standard comet
			pH >13			
			pH 12			Acellular comet
			pH >13			
			pH 12			Standard comet
			pH >13			

Fig. 3. The modes of mutagen-action, responses of standard and acellular comet assays at different pH, and expected comet images.

tion cannot act. The induction of SSBs by BLM at $\leq 1.25 \mu\text{g/mL}$ would be too low to persist until comet-preparation, which would be responsible for the negative and positive responses at $\geq 1.25 \mu\text{g/mL}$ in the stan-

dard and acellular assays, respectively.

During the base excision repair process of alkylated bases, AP sites that can form SSBs at $\text{pH} > 12.6$ are produced (5) (Fig. 3B). For alkylating agents, therefore,

DNA migration would be observed in the standard assay at $\text{pH} > 13$ but not in the acellular assay, which coincides with positive responses by alkylating agents (MNU, ENU, MMS, and EMS) in the standard assay at $\text{pH} > 13$. In the comet assay, SSBs are detected at $\text{pH} > 12$ and not only SSBs but also AP sites that are generated from AP sites can be detected at $\text{pH} > 12.6$ (5). In the acellular assay, not only BLM but also alkylating agents (MNU, ENU, MMS, and EMS) induced positive responses at $\text{pH} 12$ and $\text{pH} > 13$ and there were no differences in the response between at $\text{pH} 12$ and at $\text{pH} > 13$, which seems to indicate that DNA lesions detected at $\text{pH} 12$ are the same as those at $\text{pH} > 13$. The superiority in electrophoresis condition of $\text{pH} > 13$ is that not only DNA strand breaks but also alkali-labile sites can be detected. Alkali-labile sites are generated from initial lesions other than SSBs by cellular function such as DNA repair (5). In the acellular condition where any cellular function cannot act, alkali-labile sites could not be formed. Therefore, in the acellular condition at $\text{pH} 12$ and $\text{pH} > 13$, detected DNA lesions would be SSBs as initial lesions. Therefore, our results suggest that not only BLM but also those alkylating agents (MNU, ENU, MMS, and EMS) induce SSBs as initial lesions. In the standard assay, on the other hand, they did not lead to positive responses at $\text{pH} 12$, which seems to deny the induction of SSBs by them. Thus, the positive responses by them in the acellular assay at $\text{pH} 12$ seem to contradict to negative responses by them in the standard assay at $\text{pH} 12$. Considering that the rejoining of BLM-induced SSBs before the comet-preparation would be responsible for lower positive response by BLM in the

standard than in the acellular assays, this contradiction could be explained as follows; although initial lesions, such as SSBs, that persist until comet-preparation could be detected in the standard assay, the induction of SSBs by them is not high enough to persist until the comet-preparation.

Alkylating agents induce alkylated bases; the alkylating agents used in this study are classified into two types, unimolecular (SN-1) and bimolecular (SN-2) alkylating agents. As discussed above, positive responses in the acellular assay show the induction of SSBs as initial lesions. The positive responses in the standard assay at $\text{pH} > 13$ show the induction of DNA strand breaks and DNA lesions such as alkylated bases that can form alkali-labile sites by cellular function. (SSBs as initial lesions would not be responsible for positive responses greatly in the standard assay at $\text{pH} > 13$, since SSBs as initial lesions would not persist until comet-preparation as shown by negative responses in the standard assay at $\text{pH} 12$.) For studied alkylating agents except for MNNG, the concentration ranges where positive responses were observed were lower in the acellular assay than in the standard assay, which would suggest that the concentration range where they induce SSBs as initial lesions is lower than the concentration where they induce DNA lesions such as alkylated bases. The lowest positive concentration of SN-1 type agents (MNU and ENU) and SN-2 type agents (MMS and EMS) was $\geq 1/4$ -fold and $1/8$ -fold lower in the acellular assay than in the standard assay, respectively, which would suggest that SN-2 type agents produce SSBs easily than SN-1 type agents. In general, the SN-1 type alkylating

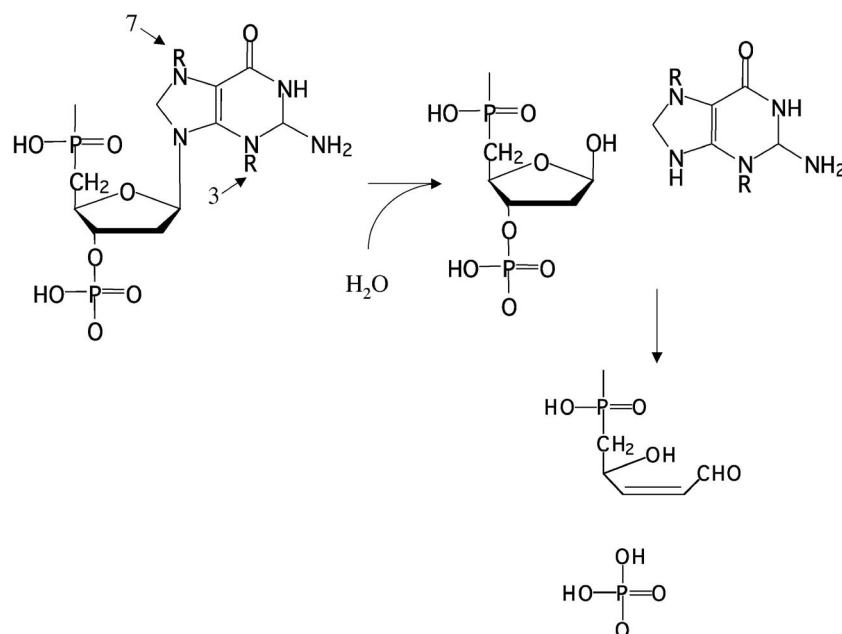


Fig. 4. Formation of SSBs from 7-alkyl purine and 3-alkyl purine.

agents produce primarily *O*-alkylated bases, whereas SN-2 type agents produce primarily *N*-alkylated bases (14). *N*-Alkylated bases such as 7-alkyl purines and 3-alkyl purines are known to be liable to develop into AP sites that can form SSBs by β -elimination (Fig. 4), which might coincide with our present discussion.

UVC led to negative responses in the acellular assay at pH 12 and pH > 13, suggesting that UVC did not induce SSBs as initial lesions. In spite of its negative response in the acellular assay at pH 12, UVC led to positive response in the standard assay at pH 12. UVC is well known to induce pyrimidine dimers that are removed by the nucleotide excision repair. SSBs are formed during the repairing process of pyrimidine dimers (Fig. 3C), which would be responsible for observed DNA migration in the standard assay at pH 12 and pH > 13. Our present results show that initial lesions other than SSBs cannot be detected in the acellular assay.

In the acellular assay, while BLM and MNU led to positive responses after ≥ 30 min treatment, MNNG did not lead to positive responses after 2 h treatment but ≥ 4 h treatment like as reported with kojic acid (10), which may suggest that SSB-induction by 2 h-exposure to MNNG is too low to be detected by this assay. Since SSBs cannot be rejoined in the acellular assay, SSBs would accumulate up to detectable level during long treatment period (≥ 4 h).

Although developing of initial lesions into alkali-labile sites through repairing events is an important factor to support the sensitivity of the comet assay, repairing events reduce its sensitivity to detect initial DNA lesion such as SSBs. As discussed above, the sensitivity of the acellular assay to detect SSBs is higher than that of the standard assay. The acellular comet assay would be usefully used to detect SSBs as initial lesions. As shown with UVC, however, the acellular assay cannot detect initial DNA lesions other than SSBs. Furthermore, as shown by Kasamatsu *et al.* (7), it cannot detect DNA lesions by mutagens, such as 4NQO, that require cellular function to show their genotoxicity.

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