

Detection of DNA Damage in Individual Cells Induced by Heavy-ion Irradiation with a Non-denaturing Comet Assay

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Investigating the biological effects of high-LET heavy-ion irradiation at low fluence is important to evaluate the risk of radiation in space. It is especially necessary to detect radiation damage induced by a precise number of heavy ions in individual cells. We thus compared the number of ions traversing a cell and the DNA damage produced by ion hits. We applied a comet assay to measure the DNA damage in individual cells. Cells attached on ion-track detector (CR-39) were irradiated with 17.3 MeV/u ¹²C, 15.7 MeV/u, 10.4 MeV/u ²⁰Ne ion and 7.2 MeV/u ⁴⁰Ar beams at TIARA, JAERI-Takasaki. After irradiation, CR-39 was covered with 1% agarose. The agarose was allowed to solidify on a glass slide, and then the electrophoresis was performed. Afterward, the CR-39 was taken off the glass slide. The agarose gel on the CR-39 was stained with ethidium bromide and the opposite side of the CR-39 was etched with a KOH-ethanol solution at 37°C. We observed that heavy ions with higher LET values induced heavier DNA damage, even with the same number of ion hits within the irradiated cells. The result indicated that the amount of DNA damage induced by one particle depended on the LET value of the heavy ions.

INTRODUCTION

In the space environment high-LET heavy ions at low fluence are a component of cosmic rays. Extended missions above the atmosphere of Earth put humans at an inherent risk due to exposure to cosmic rays. For cells exposed to high-LET heavy charged particles, DNA double-strand breaks (dsbs) have an important role in biological effects¹. At present there are many studies measuring of DNA dsbs induced by heavy ions that deposit energy at very high LET. Previously, the classical sucrose gradient centrifugation method was used to detect DNA dsbs². Recently, filter elu-

tion, conventional electrophoresis and pulsed-field gel electrophoresis³ have been used. The quantitative analysis of every approach relies on the assumption that ions hit the cells randomly, and Poisson statistics can be applied⁴. The biological effect of one particle to a cell is calculated by the analysis. This is not necessarily true, especially with heavy ions. If the number of ions that traverse a cell is precisely estimated in individual cells, one could evaluate the radiation damage induced by a precise number of heavy ions. Thus, because in this study DNA damage in individual cells was evaluated with a comet assay⁵, we established a method to simultaneously detect the number of ions traversing cellular nuclei and the DNA damage in individual cells. We compared the number of ions traversing a cell and the DNA damage detected with the comet assay.

MATERIALS AND METHODS

Cell culture

CHO-K1 cells were derived from Chinese hamster ovary, and were grown in Ham's F12 medium supplemented with 10% serum (Nichirei, Tokyo, Japan) and 0.1 mg/ml kanamycin in 10 cm diameter culture dishes, cells were incubated at 37°C in a humidified atmosphere of 5% carbon diox-

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ide and 95% air.

Irradiation

Cells were harvested after incubation for 12 hr with a medium containing 1 mM Hydroxyurea. The cells were then attached to CR-39, the particle track detector, and exposed to accelerated heavy ions⁶⁾. Accelerated heavy ions of 17.3 MeV/u ¹²C, 15.7 MeV/u, 10.4 MeV/u ²⁰Ne and 7.2 MeV/u ⁴⁰Ar were provided by the AVF cyclotron of TIARA at JAERI-TAKASAKI.

Comet assay

Immediately after irradiation, the CR-39 to which cells were attached was placed in chilled PBS. The cells were kept for 30 min on ice. The CR-39 was placed on a glass slide and then embedded in 1% agarose-LGT GP-42 (Nacalai Tesque, Kyoto, Japan). Finally, 100 μ l of 1% agarose GP-42 (Nacalai Tesque) was quickly layered. Glass slides were placed immediately in a chilled nucleus lysing solution (pH 10) of 2.5 M NaCl, 100 mM Na₄EDTA, 1% sarkosyl, 10% DMSO, and 1% Triton X-100 and kept at 4°C in the dark for 60 min. A condition that prevents the denaturation of DNA molecules (non-denaturing conditions: usually at pH values between 7 and 10) has been widely used to measure DNA damage⁷⁾. Under a neutral condition for a comet assay, the slides were placed on a horizontal gel electrophoresis platform and covered with a chilled neutral solution made up of 90 mM Tris, 2 mM Na₂EDTA and 90 mM boric acid (pH 8) for 1 hour. Electrophoresis was conducted at 4°C in the dark for 30 min at 25 V (0.89 V/cm).

The glass slides were washed with 300 mM NaOH and 1 mM Na₂EDTA, and then gently rinsed with 400 mM Tris-HCl (pH 7.5) to neutralize any excess alkali. After air drying, the CR-39 was taken off the glass slide. The agarose gel on the CR-39 was stained with 50 μ l of 0.4 μ g/ml ethidium bromide, and the opposite side of the CR-39 was etched with a KOH-ethanol solution at 37°C. Using a fluorescence microscope equipped with a green filter (Olympus, Tokyo, Japan), we examined cells at 400 magnification. The comet image was stored using a CCD camera. Then, by changing the focus with a light microscope, an image of pits etched on the CR-39 was stored using the CCD camera. Because the area of cellular nuclei was identified with the area of a high fluorescent intensity of comet image, the number of ions traversing the cellular nuclei was counted by merging both images. The comet was analyzed using the Komet software (Komet 4.0, Kinetic imaging, Ltd, UK). The tail moment, which is defined as the tail distance multiplied by

the DNA migrating into the tail^{8,9)}, was used as a parameter of the DNA damage.

RESULTS AND DISCUSSION

Fig. 1 shows a comet image of a cellular nucleus traversed by neon ions at a fluence of $0.014 \times 10^8 \text{ cm}^{-2}$. Fig. 1a shows a comet image of a cell attached to CR-39. Fig. 1b shows an image of pits etched at the opposite side of the CR-39 by changing the focus. When both images were merged, 6 etched pits were observed on the area of the cellular nucleus, that is, the area of high fluorescent intensity of the comet image. This indicates that 6 ions hit on the cellular nucleus.

Fig. 2 shows the induction of dsbs to neon-ion exposure as a function of the number of traversals. The induction of

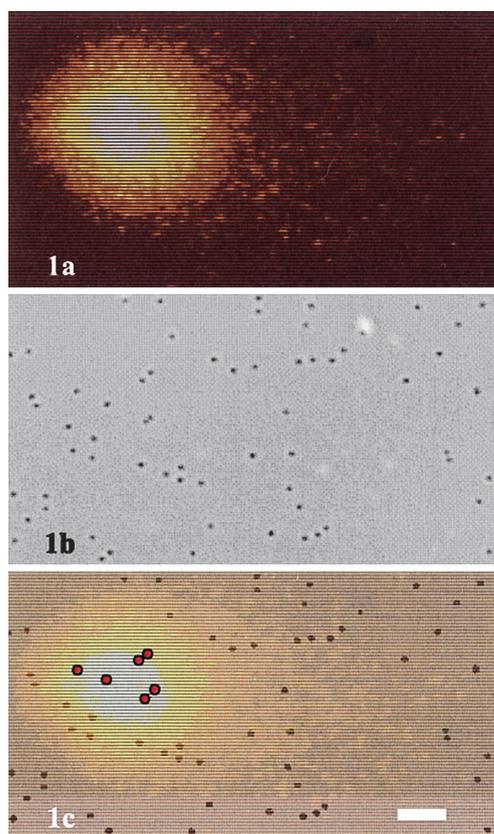


Fig. 1. Comparison of the comet image with the etched pits of particle traversals. Fig. 1a shows the comet image and Fig. 1b shows the etched pits of particle traversals at the opposite side of the CR-39 by 10.4 MeV/u neon ions at a fluence of $0.014 \times 10^8 \text{ cm}^{-2}$. A merged image of Fig. 1a to Fig. 1b can be seen in Fig. 1c. The bar indicates 10 μ m. 6 etched pits were observed on the area of a cellular nucleus that is the area of high fluorescent intensity of the comet image.

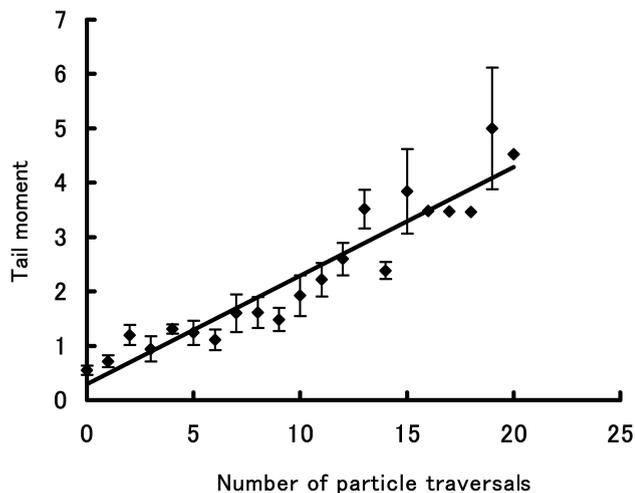


Fig. 2. Relationship between the tail moment measured by using the comet assay and the number of particle traversals of the cellular nuclei. A CHO-K1 cell was irradiated with 10.4 MeV/u neon ions. The line was fitted by a linear regression. The slope from a linear regression analysis indicates the value of the tail moment per one particle.

dsbs was evaluated as the tail moment, which was generally used to evaluate DNA damage with the comet assay. The tail moment was plotted as a function of the number of ions traversing the cellular nuclei. The tail moment increased with the number of ions traversing the cellular nuclei. The data concerning the relationship between the tail moment and the number of ions traversing the cellular nuclei was fitted by a linear regression. The slope of the fitted linear regression, calculated from the number of particle traversals of the nuclei-effect curve, indicated the induction of DNA damage per particle in the CHO-K1 cell, thus reflecting the effectiveness of the accelerated ions. In Fig. 3 the slopes calculated from the number of particle traversals of nuclei-effect curves are plotted as a function of the LET of the heavy ions. The amount of DNA damage induced by one particle increased with the LET of the heavy ions.

The molecular nature of DNA damage is determined by the spatial distribution of ionization events, which depends on the physical properties of the energy deposition and the effect of the chemical environment. The dependence on these parameters can best be evaluated using simple experimental model systems that allow an accurate quantification of DNA damage. Small plasmid molecules in solution have found wide use in radiation chemistry to study the formation of DNA strand breaks by gel electrophoresis. The cross sections that indicate the probability per one particle of inducing DNA strand breaks in the SV40 molecule increase with the LET of the ions¹⁰. In mammalian cells

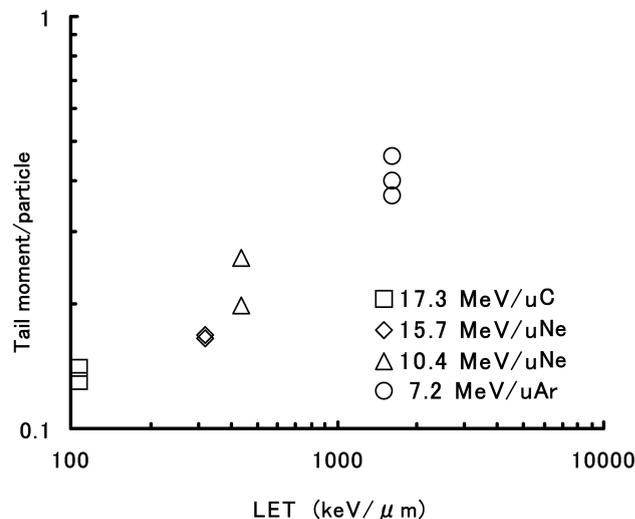


Fig. 3. Slopes as a function of the LET values. The slopes from a linear regression analysis of the number of particle traversals of the cellular nuclei-effect curves were calculated. A value of the slope and standard error is from each irradiation experiment. 17.3 MeV/u C, 15.7 MeV/u Ne, 10.4 MeV/u Ne, 7.2 MeV/u Ar.

and yeast, the cross sections of the induction of DNA strand breaks also increase with the LET of the ions, as measured by filter elution^{11,12}, sucrose sedimentation^{2,13} and gel electrophoresis^{4,14,15}. Our result also indicated that the effect per one particle of inducing DNA strand breaks increased with the LET of the ions. It was considered that LET is one of dominating parameters for evaluating the effects of the ions.

However, the cross sections saturate at a higher LET^{10,11}. The saturation observed in the cross sections as a function of LET could be explained by an increased formation of multiple damaged sites (MDS)¹⁶. Clustered dsbs, which were not resolved by the various methods used in the studies reported in the literature, may decrease the detection efficiency. This is also expected with the comet assay if it is unable to detect DNA fragments smaller than 16.5 kbp¹⁷. It was thus considered that the probability per one particle of inducing DNA strand breaks might also saturate under higher LET radiation when the comet assay is used.

The induction of dsbs is also affected by the both the track structure and the chromatin structure^{10,18,19}, and the comet assay might be sensitive to any change in both of these structures²⁰. Therefore, further studies are required to elucidate the relationship between the distribution of DNA damage induced by high-LET ions and their track structure using the comet assay.

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