Sensitive Detection of LOH Events in a Human Cell Line after C-ion Beam Exposure

SHIGEKO MORIMOTO¹, MASAMITSU HONMA² and FUMIO YATAGAI¹*

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A molecular analysis of the loss of heterozygosity (LOH) events in human cells after low-dose heavy-ion exposure could contribute to the sensitive detection of the genetic influences caused by high-LET radiation. We exposed human lymphoblastoid TK6-20C cells to 10 cGy of an accelerated C-ion (22 keV/µm) beam, and observed a 3.1-fold increase in the mutation frequency (MF) at the heterozygous thymidine kinase (TK) locus over the background level. This increase was due to the induction of TK mutants exhibiting hemizygous-type LOH. Surprisingly, the frequency of type-2 hemizygous LOHs (interstitial deletions) was about 23-fold, induced over the background level, and the LOH extent patterns of this type 2 induced after the irradiation were clearly different from that of the spontaneous background. Since hemizygous-type LOH mutants are considered to be the result of the end-joining repair of DNA double-strand breaks (DSB), C-ions may more efficiently induce DSBs than X-rays in this low-dose region. In addition, an enhanced misrepair of C-ion-induced DSBs might also account for the induction of radiation-specific hemizygous-type LOH.

INTRODUCTION

Ionizing radiation (IR) exposure induces various types of DNA lesions, including base damage, cross-linking, and DNA single- and double-strand breaks (DSBs). DSBs were originally assumed to be critical cytotoxic lesions. However, it is now accepted that misrepaired DSBs are the principle lesions of importance in the induction of chromosomal abnormalities and gene mutations¹². We recently established a gene mutation assay system whose target is the autosomal heterozygous thymidine kinase (TK) locus in the human lymphoblastoid cell line TK6⁴. This assay system detects not only intragenic point mutations, but also the loss of the functional allele (or loss of heterozygosity (LOH))⁴⁵. The presence of a diplo polymorphic frameshift mutation in the coding sequence of tk (i.e., an inactivating +1 frameshift mutation in exon 4 of the nonfunctional allele, and a silent frameshift in exon 7 of the functional allele) facilitates the classification of LOH events into two types, homozygous and hemizygous. We consider the observed homozygous and hemizygous LOHs to be the result of homologous recombinational repair (HRR) and non-homologous end-joining repair (NHER), respectively, the two major repair pathways for DSB. Furthermore, LOH events can be analyzed by 12 microsatellite markers spanning the entire chromosome 17 in TK- mutants to obtain information about the LOH tract.

Using this assay system, we found that hemizygous LOHs were induced by exposure to 2 Gy of X-rays, and that the patterns of LOH extents were different from those of spontaneously induced hemizygous LOH mutants⁶. Using a similar LOH assay technique, Giver and Grosovsky also demonstrated the same results⁷. From epidemiologic studies in human populations that are exposed to radiation from occupational, medical, and accidental sources, it has been difficult to predict the genetic influence of low-dose IR exposure. Thus, we tried an LOH analysis of TK mutants in...
TK6-20C cells that recovered following 10 cGy of X-rays, and succeeded to identify the radiation-specific hemizygous LOHs (interstitial deletions). We are interested in a further examination as to whether DSBs induced by such low-dose IR are responsible for the above-mentioned induction of hemizygous LOHs.

Since 22 keV/µm carbon-ion beam irradiation can be expected to produce DSBs more efficiently than X-ray irradiation at a similar low-dose exposure, we performed LOH analyses for TK mutants collected after the exposure of TK6-20C cells to 10 cGy of C-ions. This kind of analysis might be useful for the sensitive detection of the genetic influences caused by environmental high-LET radiation in space, although such radiation includes a variety of heavy ions with different LET. The results described below support not only the contribution of DSBs to radiation-specific LOHs, but also the development in sensitive detection methodology.

**MATERIALS AND METHODS**

To study the effect of p53 abrogation, the TK6-E6 cell line was established by introducing human papilloma virus 16 (HPV16) into parental TK6 cells. TK6-20C, which contains truncated E6, was constructed at the same time as a vector control. The sensitivity of TK6-20C to X-rays was the same as that of TK6. The cells were treated with HAT to reduce the population of background TK-deficient mutants, exposed to 10 cGy of C-ions (22 keV/µm) accelerated by the RIKEN Ring Cyclotron, and cultured for 3 days in a normal medium to express the TK phenotype. The procedures for selecting TK mutants have been previously described. The essential steps were as follows. We seeded cells into 96-well microwell plates at 20,000 cells/well in the presence of 4 µg/ml trifluorothimidine (TFT). The TFT-resistant mutants were selected after 10 days of incubation as early mutants (EMs). The plates were re-fed with TFT and incubated for another 14 days (total of 24 days) for selecting late mutants (LMs). The purpose of selecting mutants at two phases, early and late, was to make a comparison of the mutational specificity between radiation-induced and background cases easy. To reduce the selection bias for a specific type of C-ion-induced mutation, we subjected only a single mutant clone from each dish to an LOH analysis.

The LOH analysis was conducted as previously described. To distinguish whether the LOH mutants were hemizygous or homozygous for the nonfunctional allele, we performed gene dosage experiments. We measured the peak area of the nonfunctional TK alleles with an ABI310 genetic analyzer and normalized the relative ratio to that of the coamplified part of the β-globin gene on chromosome 11. To determine the extent of LOH, we also analyzed by multiple PCR the 12 microsatellites (D17S588, 784, 785, 789, 802, 807, 928, 932, 937, 1299, 1566, and THRA1) on chromosome 17.

A determination of the p53-induced cell was performed by a cell-staining method using an antibody against p53 (DO-1, Santa Cruz Biotechnology Inc). Since the cells were irradiated as a suspension culture, they were attached to the

| Treatment | Mutation Frequency (×10⁻⁶, mean±SD) | Non LOH | Hemizygous LOH Type 1 | Hemizygous LOH Type 2 | Homozygous LOH Type 1 | Homozygous LOH Type 2 | Total |
|-----------|-------------------------------------|---------|----------------------|----------------------|----------------------|----------------------|-------|
| Background | 5.7 ± 1.3                           | 43 (39) | 6 (5)                | 5 (5)                | 50 (46)              | 6 (5)                | 110 (100) |
| EM b      |                                     | 31 (28) | 0 (0)                | 3 (3)                | 0 (0)                | 0 (0)                | 34 (31) |
| LM c      |                                     | 12 (11) | 6 (5)                | 2 (2)                | 50 (46)              | 6 (5)                | 76 (69) |
| C-ion (10 cGy) | 17.9 ± 8.9 | 17 (17) | 11 (24)              | 27 (34)              | 11 (24)              | 1 (1)                | 67 (100) |
| EM b      |                                     | 14 (10) | 0 (0)                | 17 (12)              | 0 (0)                | 1 (1)                | 32 (23) |
| LM c      |                                     | 3 (7)   | 11 (24)              | 10 (22)              | 11 (24)              | 0 (0)                | 35 (77) |

a) Obtained from three independent expriments
b) EM : Early mutants
c) LM : Late mutants
d, e, f) Relative percentage for 32 EM and 35 LM mutants corresponds to 23 and 77 % of total mutants, respectively, because we analyzed the above number of mutants among the mutant collection where the number of selected mutants as LM was 3.1 times higher than that selected as EM (see text).
microscope coverslip by a cytocentrifuge before cell staining.

RESULTS

The mutation frequency (MF) at the heterozygous TK locus after exposure to 10 cGy of C-ion, 17.9±8.9×10⁻⁶ was about 3.1-times the background frequency, 5.7±1.3×10⁻⁶ (Table). The background data have already been shown in our previous report as well as the results obtained with 10 cGy X-ray exposure⁸. Although we selected 3.1 times as many LMs as EMs, we analyzed a similar number of TK mutants for each (32 EMs and 35 LMs) to characterize the nature of each type of mutation under similar levels of resolution (Table). The non-LOH type, intragenic point mutations, was almost not induced by C-ion exposure (corresponding MFs: 2.2×10⁻⁶ and 3.0×10⁻⁶ for background and C-ion, respectively). Among the EMs collected after C-ion exposure, we found a high proportion of the hemizygous LOH type (17/32). This proportion was extremely higher than that of the background (3/34). We also observed a higher proportion of hemizygous types (21/35) among LMs collected after C-ion exposure, exceeding the corresponding level of the background (8/76). As a total, the MF of hemizygous LOH type was induced by about 18-fold by C-ion exposure (0.57×10⁻⁶ to 10.4×10⁻⁶). The MF of type-2 hemizygous LOHs, considered to be radiation specific events, as described below, was about 23-times the background level (0.26×10⁻⁶ to 6.1×10⁻⁶). In contrast, the homozygous type of LOH (1/32) was rare in EM collected after C-ion exposure as well as the situation of the background (0/34). The occurrence of this event in LM was also almost not influenced by C-ion exposure (corresponding MFs: 2.9×10⁻⁶ and 4.3×10⁻⁶ for background and C-ion, respectively).

Fig. 1 shows the results of multiple (12 microsatellites) PCR analysis for the above-mentioned LOH mutants. A type-1 event (terminal deletion) for both hemizygous and homozygous LOH was defined as a pattern in which the

| Types of LOH | Background | Markers on Chromosome 17 | C-ion (10 cGy) |
|-------------|------------|--------------------------|---------------|
|             | E M        | L M                      | E M           |
| Hemi LOH    |            |                          |               |
| Type 2      |            |                          | Type 2        |
| Type 1      |            |                          | Type 1        |
| Homo LOH    |            |                          |               |
|             |            |                          |               |

Fig. 1. Extent of LOH on chromosome 17. Illustrations are separately shown for TK-deficient mutants obtained at an early period (EM) and a late period (LM) (see text). In the mutant marked with an asterisk, the D17S1566 marker region (not shown in this figure and located in the short arm of chromosome 17) could not be amplified by PCR.
deleted or exchanged chromosome part extended to the telomere marker, D17S928, of the long arm. In a type-2 event, this part did not reach the marker. All patterns of the 17 hemizygous LOHs in EMs were type 2 (interstitial deletions). Among them, 3 events of smallest deletion at the TK locus had the same patterns that we recovered in the background spectrum. We therefore considered most of the type-2 hemizygous LOHs to be the result of C-ion exposure. Among hemizygous LOHs in LM s, we recovered types 1 and 2 at almost the same frequency (11/21 and 10/21, respectively). Interestingly, we found the following unique characteristics in the type-2 events of hemizygous LOH in LM s: (1) the smallest deletion observed frequently as a background mutation was not recovered in this category, and (2) more than half (6/10) of this category showed deletion sizes which were larger than those recovered in EM s. All homozygous LOHs in LM s were of type 1.

DISCUSSION

Although a 10 cGy dose of X-rays caused a 1.2-fold increase in the TK mutation frequency over the background level, this enhancement was not statistically significant (p=0.39). The same low-dose of the C-ion, however, resulted in a 3.1-fold number of mutants (p=0.04). Furthermore, an LOH analysis clearly revealed that the characteristics of the LOH events recovered after C-ion exposure were different from those for the unirradiated control. The proportion of hemizygous LOHs among the total TK mutants increased from a control value of 10 % (MF; 0.57×10⁻⁶) to 58% (MF; 10.4×10⁻⁶) after C-ion exposure. This increase corresponds to about 18-fold induction in MF; this level is not very much different between type 1 (15-fold) and type 2 (23-fold). However, a further multi-locus PCR analysis for this type of LOH clearly indicated that type-2 hemizygous LOH mutants were specific to radiation exposure. Indeed, such an analysis demonstrated that LOHs after radiation exposure extended outside the TK locus, in contrast to the small deletion-type that was restricted to the TK locus, and frequently recovered in the background control. The radiation-specific type-2 hemizygous LOHs (interstitial deletions) can be considered to be a sensitive indicators of the genetic influences of ionizing-radiation exposure. Surprisingly, we observed this radiation-specific LOH pattern in more than half of these hemizygous-type LOH events. In fact, the MF of such radiation-specific interstitial deletions after C-ion exposure (excluding the spontaneously recovered one from type-2 hemizygous LOH), 5.7×10⁻⁶, is 5.2-fold higher than the corresponding MF after the same dose of X-ray irradiation, 1.1×10⁻⁶. Thus, the frequency and LOH extent of such type-2 hemizygous LOHs could reflect differences in the radiation quality. A more frequent recovery of this type of LOH after C-ion exposure, compared with X-ray exposure, suggests that DSBs are efficiently produced by densely deposited energy along the C-ion track. Under the present irradiation condition of 10 cGy C-ion, the average number of C-ion traversals through the cell nucleus was calculated to be 1.1. This result from a simple calculation might also support the efficient production of DSB.

There are a considerable number of reports about the cross section of LET-dependent DNA strand breaks, SSB and DSB. In fact, the ratio of DSB to SSB in SV40 DNA was found to increase with increasing LET of the C-ion in the range of 17 to 220 keV/µm8, and such a ratio after 22 keV/µm carbon-ion beam irradiation seemed to be about 2-fold higher than that after X-ray irradiation. The efficient induction of DSB in primary human skin fibroblasts, compared to X-ray irradiation, has already been shown after irradiation with ³H and ⁴He ions in the LET range of 8 to 124 keV/µm13. However, DSB production by 17 keV/µm of C-ion irradiation was reported to be not very efficient in mammalian cells (RBE 1.1)13. Since this RBE value was calculated from measurements of DSB by constant-field gel electrophoresis within the dose-range of 50 Gy, it might not reflect the possible efficient-production of DSB by 22 keV/µm of C-ion in a low-dose range, such as 10 cGy. The proportion of p53 induced cells after low-dose exposure of 22 keV/µm C-ions and X-rays was determined by an immunofluorescence staining of cells (Fig. 2). The results indicate

![Fig. 2. Proportion of p53 induced cells.](https://academic.oup.com/jrr/article-abstract/43/Suppl/S163/1107969)
that a higher efficiency of damage production occurs by 10 cGy C-ion compared to that by the same dose of X-rays. Although this is not a direct measurement of DSB production, a higher efficiency can be expected from this result. Clustered DNA damage, two or more closely spaced lesions (strand breaks, abasic sites, or oxidized damages) on opposing strands, was induced in human cells by 0.1 to 1.0 Gy of 1 GeV Fe-ion irradiation\(^1\). We suspect that C-ion-induced clustered DNA damage is as efficient as Fe-ion irradiation, because we observed large γH2AX-foci formation, reflecting clustered damage by the 1000 keV/µm Fe-ion\(^2\), but is not efficient by the 22 keV/µm C-ion (data not shown). Although the DSBs produced by 22 keV/µm C-ions could not efficiently cause the clustered damage, they might be difficult to repair compared to the X-ray case. As a result, an enhanced misrepair of such damage could account for the induction of hemizygous-type LOH. We also cannot neglect the possibility that the DSBs which occurred spontaneously and/or were induced indirectly, might act as one of the deletion ends, resulting in an enhanced induction of specific LOH.

The present data strongly suggests that the molecular analysis of the loss of heterozygosity (LOH) events in human cells after low-dose heavy-ion exposure contributes to the estimation of the genetic influence of high-background high-LET radiation. Taking into consideration of bystander effects\(^3\) and gene expression\(^4\), we would like to promote this line of study.

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