Analysis of Unrejoined Chromosomal Breakage in Human Fibroblast Cells Exposed to Low- and High-LET Radiation

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High-LET radiation / Chromosome aberration / Telomere / Unrejoined breaks

Reported studies of DNA breakage induced by radiation of various qualities have generally shown a higher fraction of unrejoined residual breaks after high-LET exposure. This observation is supported by the argument that high-LET radiation induced DNA breaks that are more complex in nature and, thus, less likely to be repaired. In most cases the doses used in these studies were very high. We have studied unrejoined chromosome breaks by analyzing chromosome aberrations using a fluorescence in situ hybridization (FISH) technique with a combination of whole chromosome specific probes and probes specific for the telomere region of the chromosomes. Confluent human fibroblast cells (AG1522) were irradiated with γ rays, 490 MeV/nucleon Si, or with Fe ions at either 200 and 500 MeV/nucleon, and were allowed to repair at 37°C for 24 hours after exposure. A chemically induced premature chromosome condensation (PCC) technique was used to condense chromosomes in the G2 phase of the cell cycle. Results showed that the frequency of unrejoined chromosome breaks was higher after high-LET radiation, and the ratio of unrejoined to misrejoined chromosome breaks increased steadily with LET up a peak value at 440 keV/µm.

INTRODUCTION

It has been suggested that high-LET radiation produces more severe or complex DNA double strand breaks (DSB) that are less likely to repair than DSB produced by low-LET radiation1). Studies of radiation-induced DNA breaks using pulse-field gel electrophoresis have indeed shown that high-LET radiation produced more residual DNA double strand breaks (DSB) than low-LET radiation2). Since chromosomal breaks are associated with the DSB, one would expect more unrejoined residual chromosomal breaks after high-LET radiation exposure. However, caution should be paid by the following reasons when applying this argument to chromosomal breaks. Firstly, chromosome aberrations observed in metaphase do not directly represent the damage and damage repair occurring in interphase. If cells are exposed in the G0/G1 phase of the cell cycle and chromosomes are analyzed in mitosis, cell cycle effects have to be considered because cells with chromosome breaks may be less likely to reach mitosis than normal cells, as has been shown in the case of cells with certain types of chromosome aberrations3–5). Secondly, terminal deletions and incomplete chromosomal exchanges resulting from unrejoined chromosomal breaks can sometimes be misidentified. It is difficult to distinguish a terminal deletion from an interstitial deletion using the techniques commonly used for chromosome analysis. It has also been known that a majority of the incomplete exchanges analyzed by the FISH technique could be falsely identified, since some chromosomal exchanges are below the level of resolution6–11).

Premature chromosome condensation (PCC) techniques provide a tool for analyzing chromosomal aberrations before the cell reaches mitosis. One of the PCC techniques that is becoming popular condenses G2 chromosomes using the protease inhibitor calyculin-A12,13). Durante et al.3) found similar frequencies of aberrations in chromosomes condensed using calyculin-A and in chromosomes condensed in G1 phase using the cell fusion PCC technique, but lower frequencies were observed in chromosomes collected at

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metaphase, apparently due to the effect of cell cycle delay or cell cycle block. It has also been reported that cells with certain types of aberrations are less likely to reach mitosis than cells with other types of aberrations3–5).

Unrejoined chromosomal breaks can be accurately identified using telomere probes, since unrejoined breaks exhibit no telomere signals at the end of the chromosome fragment6–11). A round 20% of low-LET induced chromosome aberrations in human lymphocytes were identified as incomplete exchange using FISH painting alone, but when telomere probes were used in conjunction with FISH painting, the percentage of true incomplete exchanges was found to be about 3%6–8). The percentage of true incomplete exchanges induced by high-LET radiation is less certain, however. For metaphase chromosomes, Fomina et al. observed that similar percentages of aberrations were incomplete in human lymphocytes after in vitro exposure to gamma rays or neutrons11). Our earlier study of incomplete exchanges in lymphocytes exposed to 1 GeV/nucleon Fe ions (LET = 140 keV/µm) was hampered by large statistical uncertainties and is inconclusive as to whether the percentage of incomplete exchanges is higher after high-LET exposures44). These results were, however, derived from analysis of metaphase chromosomes.

We report here results of a study of unrejoined chromosomal breaks in interphase chromosomes using a combination of FISH painting with whole chromosome specific probes and telomere probes. Normal human fibroblast cells were exposed in vitro to gamma rays or high-energy charged particles. The cells were allowed to repair for 24 hours after irradiation and, thus, the unrejoined breaks were supposed to represent residual breaks. Interphase chromosomes were condensed using the chemically induced PCC technique with calyculin-A12,13).

**MATERIALS AND METHODS**

**Cell culture and irradiation**

Normal human fibroblast cells, AG1522, were obtained from the NIA cell repository, grown in α-minimum essential medium with 10% calf serum, and irradiated in the confluent state. After exposure, cells were allowed to repair at 37°C for 24 h before they were transferred from a T-25 flask to a T-75 flask. After further incubation at 37°C for 32 hours, chromosomes were condensed by incubating in calyculin-A at a concentration of 50 nM (Waco Chemicals, Japan) for 30 minutes. The cells were swollen in 0.075 M KCl solution at 37°C for 20 minutes and fixed in methanol/acetic acid (3:1 vol/vol) fixative solution.

Cells were exposed to approximately equitoxic doses of radiations of different ionization densities. For low-LET exposure, the cells were exposed to 137Cs γ rays at a dose rate of 10 Gy/min. High-LET ion particles, generated at Heavy Ion Medical Accelerator in Chiba (HIMAC), Japan, included 490 M eV/nucleon Si, and 200 and 500 M eV/nucleon Fe ions. The dose rates varied between 0.5 and 1 Gy/min.

**Fluorescence In Situ Hybridization (FISH)**

Chromosome spreads were dropped onto clean slides and after aging at room temperature for 7 days, they received enzyme treatment similar to that used by Boei et al.9) and Deng et al.10). Briefly, slides were washed in 1X PBS for 5 minutes and dehydrated in 70%, 85% and 100% ethanol for 2 minutes each at room temperature. Slides were then incubated in RNase A (100 µg/ml) in a humidified chamber at 37°C for 30 minutes, washed with 2XSSC and PBS, and further treated with pepsin (0.05% in 10 mM HCl) for 5 minutes at 37°C. After washing with PBS and MgCl2 (50 mM in PBS), slides were fixed in 1% formaldehyde, washed, and dehydrated in 70%, 85% and 100% ethanol.

Enzyme treated chromosomes were then denatured in 70% formamide for 2 minutes at 72°C, immediately dehydrated in cold 70%, 85% and 100% ethanol for 2 minutes each and air-dried. Hybridization of the telomere and whole chromosome probes was performed in two steps. The hybridization mixture for telomere detection was prepared by combining 7 µl hybridization buffer with 2 µl (5 µg/ml) fluorescein-labeled PNA telomere probe (PerSeptive Biosystem, Framingham, MA, USA). The telomere probe mix was denatured at 72°C for 5 minutes, applied to the slide and covered with a 2 X 2 cm coverslip. Slides were then incubated at room temperature for 3 hours, washed briefly with 2 X SSC at room temperature and dehydrated with 70%, 85% and 100% ethanol for 2 minutes each. The whole chromosome probes for chromosomes #3 and #5 labeled in orange were then denatured at 72°C for 5 minutes and applied to the slide. Chromosomes were hybridized at room temperature overnight. Slides were washed at 37°C twice in 50% formamide for 15 min. each, once in 2 X SSC for 5 minutes and once in 0.1% NP40 in 2 X SSC for a further 5 minutes. DAPI was applied as counterstain.

**Classification of aberrations**

Classification of chromosome aberrations was similar to the system used in our previous study4). When both regions of a broken painted chromosome were visibly translocated to unpainted chromosomes, the exchange was scored as complete. Since it is difficult to identify centromeres in the
PCC chromosomes without using a centromere probe, this
classification of complete exchanges included complex
exchanges where three or more chromosome breaks may be
involved. False incomplete exchanges displaying telomere
signals on both ends of the painted fragment were included
with the complete exchanges. A true incomplete exchange
or a terminal deletion was identified when a painted chro-
mosomal fragment displayed telomere signals at only one
end. The reader is referred to Ref. 8 for a schematic diagram
of different types of aberrations.

In the present study we also compared unrejoined and
misrejoined chromosomal break ends for each type of rad-
iation. A terminal deletion was scored when a painted frag-
ment had two unrejoined ends, while a true incomplete
exchange had one unrejoined break end and one misre-
joined end. A complete exchange contained two misre-
joined ends and insertions could have more than two misre-
joined ends depending on the number of DSB involved in
the painted chromosomes. Interstitial deletions that did not
show telomere signals were excluded from this analysis.
The ratio \( N_{\text{unrejoined}}/N_{\text{misrejoined}} \) is presented in the Results,
where \( N_{\text{unrejoined}} \) and \( N_{\text{misrejoined}} \) are the number of unrejoined
and misrejoined chromosome break ends, respectively.

RESULTS

Aberrations in chromosomes #3 and #5 from AG1522
cells after irradiation with \( \gamma \), 490 MeV/nucleon Si, and 200
and 500 MeV/nucleon Fe ions are shown in Table 1. The
dose selected for each radiation type corresponds to roughly

| Radiation | LET (keV/µm) | Dose (Gy) | Cells scored | Complete exchange | True Incomplete exchange | Insertion | Terminal deletion and centric ring | Terminal deletion | Ratio of unrejoined to misrejoined break ends |
|-----------|--------------|-----------|--------------|-------------------|-------------------------|-----------|-----------------------------------|-------------------|--------------------------------------------|
| Gamma     | 0.6          | 6         | 105          | 32                | 2                       | 0         | 11                                | 1                 | 0.06 ± 0.03                                |
| Si        | 490 MeV/u    | 55        | 2            | 187               | 39                      | 5         | 0                                 | 12                | 0.08 ± 0.03                                |
| Fe        | 500 MeV/u    | 200       | 2            | 93                | 40                      | 8         | 7                                 | 13                | 0.12 ± 0.03                                |
| Fe        | 200 MeV/u    | 440       | 3            | 124               | 55                      | 18        | 10                                | 12                | 0.16 ± 0.03                                |

**Table 1.** Aberrations in Chromosomes #3 and #5 in human fibroblast cells exposed to radiation of various qualities. The numbers in parenthesis indicate the frequency and the standard deviation.

**Fig. 1.** Frequency of unrejoined chromosomal break ends in Chromosomes #3 and #5 per Gy of dose. According to the classification here, an incomplete exchange produced one unrejoined break end and a terminal deletion produced two unrejoined break ends in the painted chromosome.

**Fig. 2.** Frequency of total interchromosomal exchanges per Gy of dose in Chromosomes #3 and #5.
10% survival. Previously, we found 0.003 aberrations in chromosome #4/cell in the unirradiated AG1522 cells. Thus, the frequency of aberrations would be insignificant at 0 dose in comparison to the frequency of aberrations at other doses in the present study. The frequency of unjoined break ends per cell per Gy as a function of LET, shown in Fig. 1, was much higher for the Fe irradiated samples compared to samples irradiated with 490 MeV/u Si ions. The frequency of unjoined breaks for 500 was similar to that for 200 MeV/u Fe ions. The total exchange frequency per Gy is shown in Figure 2. Similar to RBE for most biological endpoints, the total interchromosome exchange frequency per Gy increased with increasing LET, reached a peak around 100 keV/µm and decreased at higher LET values. The ratio of unjoined to misrejoined break ends, shown in Table 1 and in Fig. 3 as a function of LET, increased steadily as the LET value increased.

DISCUSSION

By using telomere probes that allow accurate identification of unjoined chromosomal breaks, we found that the fraction of unjoined breaks was higher for high-LET than for low-LET in chromosomes condensed using calyculin-A. The present results support the theoretical prediction that high-LET radiation induces more severe DSB damage, which is less likely to repair. The LET value in the present study covers a range between 0.6 and 440 keV/µm. Comparison between the 200 keV/µm and 440 keV/µm LET points shows that the number of total exchanges per Gy decreased for the higher LET values, but the number of unjoined breaks per Gy remained similar. Over the LET range of the present study, the ratio of unjoined to misrejoined chromosomal breaks increased steadily with LET.

For most biological endpoints, the RBE increases with LET, reaches a peak around 100 keV/µm and declines for higher LET values. The exact LET value of the RBE peak varies depending on the type of high-LET ions and on the biological endpoint. In the present study, the RBE for total interchromosome exchanges was the highest for 500 MeV/u Fe ions (LET = 200 keV/µm), which is in agreement with the results reported in the literature. However, of the total chromosome aberrations, the fraction of unjoined breaks appeared to be greater for higher LET values. The RBE for unjoined breaks will likely to peak at a higher LET value, but whether the ratio of unjoined to misrejoined break ends will decline at LET values beyond 440 keV/µm remains to be determined.

After the proposal by Brenner and Sachs to use the ratio of dicentrics to centric rings as a marker for radiation quality, suggestions for other yield ratios such as the ratio of complex to simple exchanges as potential biomarker for the quality of radiation have also been made. While some of the yield ratios show a difference between low- and high-LET, it is not clear whether those ratios hold for higher LET values where the RBE for most biological endpoints is below its peak value. Results of the present study showed that the ratio of unjoined to misrejoined chromosomal breaks can potentially be used as a biomarker for the quality of radiation at early times post-irradiation.

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