Analysis of Heavy ion-induced chromosome aberrations in human fibroblast cells using in situ hybridization

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ABSTRACT

Confluent human fibroblast cells (AG1522) were irradiated with γ rays, 490 MeV/nucleon Si, or with Fe ions at either 200 or 500 MeV/nucleon. The cells were allowed to repair at 37 $^{\circ}$C for 24 hours after exposure, and a chemically induced premature chromosome condensation (PCC) technique was used to condense chromosomes in the G2 phase of the cell cycle. Unrejoined chromosomal breaks and complex exchanges were analyzed in the irradiated samples. In order to verify that chromosomal breaks were truly unrejoined, chromosome aberrations were analyzed using a combination of whole chromosome specific probes and probes specific for the telomere region of the chromosome. Results showed that the frequency of unrejoined chromosome breaks was higher after high-LET radiation, and consequently, the ratio of incomplete to complete exchanges increased steadily with LET up to 440 keV/μm, the highest LET value in the present study. For samples exposed to 200 MeV/nucleon Fe ions, chromosome aberrations were analyzed using the multicolor FISH (mFISH) technique that allows identification of both complex and truly incomplete exchanges. Results of the mFISH study showed that 0.7 and 3 Gy dose of the Fe ions produced similar ratios of complex to simple exchanges and incomplete to complete exchanges, values for which were higher than those obtained after a 6 Gy γ exposure. After 0.7 Gy of Fe ions, most complex aberrations were found to involve three or four chromosomes, indicating the maximum number of chromosome domains traversed by a single Fe ion track.

INTRODUCTION

Energetic heavy ions of high-LET induce chromosome aberrations that are both quantitatively and qualitatively different from chromosome aberrations induced by low-LET radiation (1, 2), due perhaps to both the severity and the spatial distribution of the DNA damage induced by this type of radiation. High-LET radiation is believed to produce more severe or complex DNA breaks that are less likely to repair than DNA breaks produced by low-LET radiation (3), and studies of DNA breaks using pulse-field gel electrophoresis have indeed shown that high-LET radiation induced more residual DNA double strand breaks (DSB) than low-LET radiation (4). A higher yield of residual chromatin breaks was also reported in human fibroblast cells exposed to high-LET radiation in the G2 phase of the cell cycle (5). Since chromosomal breaks are associated with the induction of DSB, more unrejoined residual chromosomal breaks, in the form of terminal deletions or incomplete reciprocal exchanges, would be expected after high-LET radiation exposure. In fact, the ratio of incomplete to complete exchanges has been suggested as a possible biomarker of radiation quality (6).

However, many of the incomplete reciprocal type exchanges analyzed using FISH with whole chromosome specific probes could be falsely identified, since some chromosomal exchanges are likely to be below the level of detection for this technique (7). It is possible to identify truely terminal deletions and incomplete exchanges using a combination of FISH with whole chromosome specific probes and probes that hybridize specifically to the telomere region of the chromosome (8-11). Around 20% of low-LET induced chromosome aberrations in human lymphocytes have been identified as incomplete using FISH painting alone. However when telomere probes were used in conjunction with FISH painting, the percentage of true incomplete exchanges was found to be only about 3% (8-11). Although reports using FISH painting alone have demonstrated that ratios of complete to incomplete exchanges are LET dependent, few studies have confirmed that high-LET induced more truly unrejoined chromosomal breaks, and therefore it is still unclear whether incomplete exchanges can be used as a marker of radiation quality. In fact, Formina et al. have shown that fractions of truly incomplete exchanges were actually similar in lymphocytes after exposure to X rays or high-LET neutrons (11, 12).

High-LET radiation induces ionizations and DSB that are densely distributed around the track of the primary particle, and this produces more complex type chromosome exchanges involving

exchanges of three or more chromosomal breaks than equivalent doses of low-LET radiation (13). Studies of complex aberrations have been reported using FISH with one or two whole chromosome specific probes(1, 14-16). However with this technique it is not always possible to determine all the chromosomes involved in a complex exchange. The recently developed multicolor FISH (mFISH) technique allows each human chromosome pair within an individual cell to be painted a different color, and this has proved particularly useful for determining the true complexity of chromosome exchanges (17-19) since all chromosomes can be visualized simultaneously and the exact number of chromosomal breaks can be resolved .

In the present paper, we report the results of a study of chromosome aberrations in human fibroblast cells exposed in vitro to γ rays, 490 MeV/nucleon Si, or Fe ions of 200 or 500 MeV/nucleon. Chromosome aberrations were analyzed using two techniques; a combination of FISH painting with whole chromosome specific probes and telomere probes, and, for 200 MeV/nucleon Fe ion irradiated samples, the mFISH technique. Human fibroblast cells are frequently used in the study of radiation-induced chromosome aberrations (14, 20).

In addition to comparing absolute frequencies of low and high-LET induced chromosome aberrations , we focus here on the relative frequencies of simple to complex, and incomplete to complete exchanges; two potential markers that have been suggested for assessing the radiation quality. We also attempt to identify truly incomplete exchanges using mFISH. It has been suggested (Lucas, J. N., private communication) that truly incomplete exchanges can be resolved by studying only the exchanges between two different colored FISH painted chromosomes, where true incompletes would result in a bicolor chromosome accompanied by two broken fragments, one of each color. With mFISH this criteria can be used for assessing incomplete exchanges in all chromosomes simultaneously, and the likelihood of detecting an incomplete exchange is now much higher than in cases where only two specific chromosomes are considered.

MATERIALS AND METHODS

Cell culture and irradiation

Normal human fibroblast cells, AG1522, obtained from the National Institute of Aging (NIA) cell repository, were 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

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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.

For telomere analysis, cells exposed to approximately equitoxic doses of radiations of different ionization densities were used. For low-LET exposure, the cells were exposed to ^{137}Cs γ rays at a dose rate of 10 Gy/min. High-LET ion particles, generated at the Heavy Ion Medical Accelerator in Chiba (HIMAC), Japan, included 490 MeV/nucleon Si, and 200 and 500 MeV/nucleon Fe ions. The dose rates varied between 0.5 and 1 Gy/min. For mFISH analysis, samples exposed to 6 Gy γ rays, and 200 MeV/nucleon Fe ions at 0.7 and 3 Gy were used.

Slide preparation Fluorescence In Situ Hybridization with telomere probes (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 $MgCl_2$ (50 mM in PBS), slides were fixed in 1% formaldehyde, washed, and dehydrated in 70%, 85% and 100% ethanol.

Fluorescence In Situ Hybridization with telomere probes (FISH)

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 2XSSC 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 with spectrum orange were then denatured at 72 °C for 5 minutes and applied to the slide. Chromosomes were hybridized overnight at room temperature . Slides were washed at 37 °C twice in 50% formamide for 15 mintes 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.

mFISH analysis

Chromosome spreads were dropped onto clean slides. After aging at room temperature for 2 weeks, chromosomes underwent enzyme treatment as described above. 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. Slides were then hybridized with Spectra Vysion[TM] probes (Vysis, Downers Grove, IL), following the basic protocol recommended by the manufacture.

Classification of aberrations

Classification of chromosome aberrations was similar to the system used in our previous study (21). 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 as complete. A true incomplete exchange or a terminal deletion was identified when a painted chromosomal fragment displayed telomere signals at only one end. The reader is referred to Ref. 21 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 radiation. A terminal deletion was scored when a painted fragment had two unrejoined ends, while a true incomplete exchange had one unrejoined break end and one misrejoined end. A complete exchange contained two misrejoined ends and insertions could have more than two misrejoined 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.

With mFISH analysis chromosome aberrations invoving a bicolor chromosome accompanied by two broken fragments, one of each color, were classified as truly incomplete. This classification can be extended to exchanges involving more than two DSB. An example of a true incomplete among Chromosomes #1, #10 and X is shown in Fig. 1.

RESULTS

Telomere analysis

Aberrations in chromosomes #3 and #5 from AG1522 cells after irradiation with γ, 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 10% survival. Previously, we found 0.003 aberrations in chromosome #4 /cell in the unirradiated AG1522 cells. Thus, the background frequency of aberrations would be insignificant in comparison to the frequency of aberrations induced by the doses used the present study. The frequency of complete exchanges per Gy peaked at 200 keV/μm, while the frequencies of true incompletes per Gy were similar for the 500 and 200 MeV/nucleon Fe ion exposures. Thus the ratio of incomplete to complete exchanges, as well as the ratio of unrejoined to misrejoined break ends, were higher for 200 MeV/nucleon Fe ions, as shown in Figure 2. The frequency of total exchanges per Gy (the sum of all aberrations identified) peaked at 200 keV/micron of LET, similar to most other biological endpoints.

mFISH analysis

The yield of exchanges induced by 6 Gy γ, and 0.7 and 3 Gy Fe ions are listed in Table 2 along with the ratios of complex to simple exchanges (C ratio following the suggestion of M. Cornforth), and the ratio of incomplete to complete exchanges. It is seen that both ratios were significantly different between the samples exposed to 200 MeV/nucleon Fe ions and 6 Gy γ rays.

Although the C ratios were similar for both doses of Fe ions, complex exchanges induced by 3 Gy Fe ions were more complex in nature and involve more chromosomal breaks than those induced by 0.7 Gy Fe ions. Figure 4 shows the number of chromosomes involved in reciprocal and complex exchanges. In the 3 Gy Fe irradiated samples, we identified one complex exchange involving eight chromosomal breaks, while no complex exchanges involving more than four chromosomal breaks were found in the samples exposed to 0.7 Gy Fe ions. The maximum number of chromosomes

involved in a complex exchange was 4 and 7 in 0.7 and 3 Gy Fe irradiated samples, respectively. The most complex type exchange identified in the 6 Gy γ irradiated samples involved six chromosomal breaks. Since human fibroblast cells are flat in nature with a thickness of 1-2 μm, the observation that most complex aberrations involved no more than four chromosomes in 0.7 Gy Fe ion samples suggests that a maximum of four chromosome domains is perhaps the most that a single track of Fe ion could traverse. The small number of complex exchanges involving five or more chromosomes observed in the 3 Gy Fe exposed samples were perhaps due to recombination of chromosomal breaks induced by more than one ion.

DISCUSSION

Chromosome aberrations in human fibroblast cells exposed to both low- and high-LET radiation were analyzed using two in situ hybridization techniques. Using telomere probes that allow accurate identification of unrejoined chromosomal breaks, we found that the fraction of unrejoined 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 values in the present study cover a range of 0.6 to 440 keV/μm. When comparing the 200 keV/μm and 440 keV/μm LET points, the total number of exchanges per Gy decreased at the higher LET value, but the number of unrejoined breaks per Gy remained similar. The ratio of unrejoined to misrejoined chromosomal breaks, as well as the ratio of incomplete to complete exchanges, increased steadily over the LET range used in the present study . The mFISH analysis also showed a higher ratio of incomplete to complete exchanges in samples irradiated with 200 MeV/nucleon Fe ions than with γ rays. The present results confirmed that the yield ratios associated with unrejoined chromosomal breaks (such as the ratios of unrejoined to misrejoined breaks or of incomplete to complete exchanges) are potential biomarkers for the quality of radiation.

The present results were in disagreement with reported studies of human lymphocytes exposed in vitro to X-rays and high-LET neutrons (11, 12), however. Since the DNA in cells exposed to neutrons is directly damaged by the secondary protons, it is possible that the severity of DSB in neutron irradiated cells is similar to that induced by low-LET proton exposures. The samples

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analyzed in the present study were condensed using Calyculin-A. It has been shown that chromosome aberration frequencies were different for exposed samples collected using Calyculin-A and collected at mitosis, particularly for high-LET, since damaged cells may not reach mitosis or may reach mitosis at a later time (16, 22, 23). It is likely that cell cycle perturbations may also affect the yield ratios. It has been shown that the ratio of inter- to intrachromosome exchanges (The F ratio) was similar for low- and high-LET samples analyzed in metphase, but was different in samples collected using Calyculin-A (24). Therefore, the LET dependent relationship between the fraction of unrejoined chromosomal breaks shown in the present study for PCC analysis, might not necessarily be the observed in metaphase samples.

mFISH analysis also showed a higher ratio of complex to simple exchanges for 200 MeV/nucleon Fe ions compared with γ rays. High-LET radiation produces ionizations that are densely distributed around the track of the primary particle. The spatial distribution of DNA breaks for high-LET is thus different from the random distribution usually assumed for low-LET radiation. Spatially, chromosomes in interphase are localized in individual domains and closer DSB are more likely to interact than distant ones. The spatial consideration alone would predict that high-LET radiation produces more complex aberrations than low-LET radiation (13). Hence, the ratio of complex to simple aberrations may be used as a biomarker for the quality of radiation. Data at other LET points are needed, however, in order to determine whether the C ratio continues to increase with high LET values.

Although high-LET radiation induces a greater fraction of complex to simple aberrations, the C ratio is still far less than values reported for lymphocytes exposed to similar doses of both low and high-LET radiation (17-19). The absolute frequency of radiation-induced chromosome aberrations in human fibroblast cells is also less than values reported for human lymphocytes (25). The geometry of the cell nucleus and the compactness of chromosomes appear to play a role in these differences in radiosensitivity (25). The lower yield of complex aberrations in fibroblasts can also be attributed in part to the flatness of the cell nucleus. As shown in the present study, a charged particle would perhaps pass through no more than four chromosome domains in fibroblasts, whereas more chromosome domains are likely to be traversed in lymphocytes (18), resulting in complex exchanges involving more chromosomal breaks.

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Figure captions

Fig. 1. Examples of true incompletes and false incompletes in chromosomes painted with mFISH. The true incomplete involves exchanges among Chromosomes #1, #10 and X, while the false incomplete involve Chromosomes #1 and #14.

Fig. 2. Ratio of unrejoined to misrejoined chromosomal break ends and of incomplete to complete exchanges in Chromosomes #3 and #5 analyzed with telomere probes. A complete exchange contained two misrejoined ends and insertions may have more than two misrejoined ends depending on the number of DSB in the painted chromosomes involved

Fig. 3. Ratios of complex to simple and of incomplete to complete exchanges in human fibroblast cells exposed to γ rays and 200 MeV/nucleon Fe ions, analyzed with mFISH.
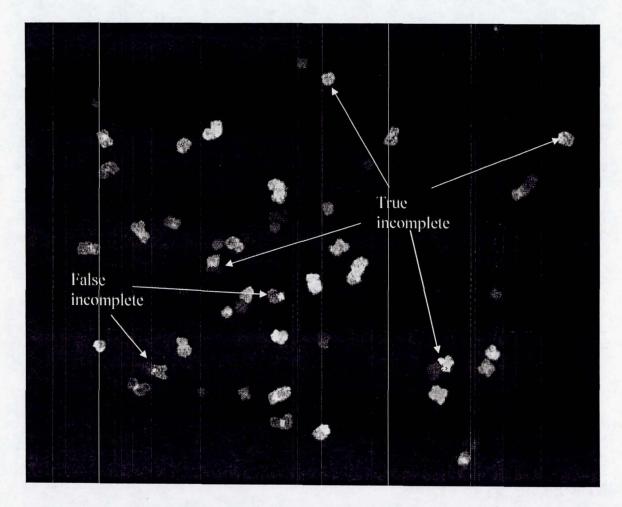
Fig. 4. Number of chromosomes participated in exchanges. The maximum numbers of chromosomes were 6, 4 and 7 for 6 Gy γ, 0.7 Gy Fe and 3 Gy Fe ions, respectively.
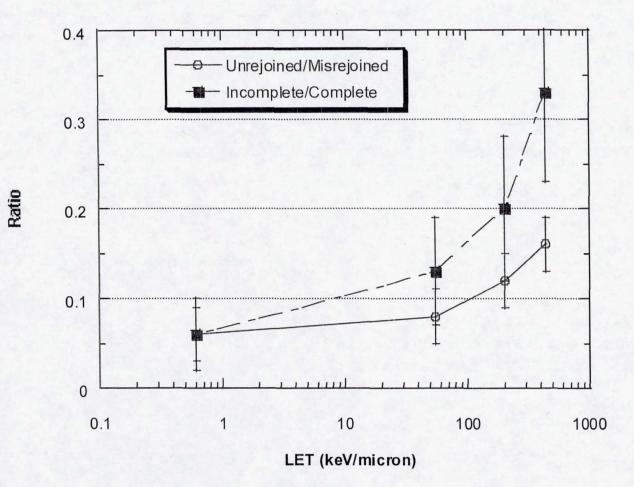
Table 1. Aberrations in Chromosomes #3 and #5 in human fibroblast cells exposed to radiation of various qualities. The numbers in parenthesis indicate the frequency per Gy of dose and the standard deviation.
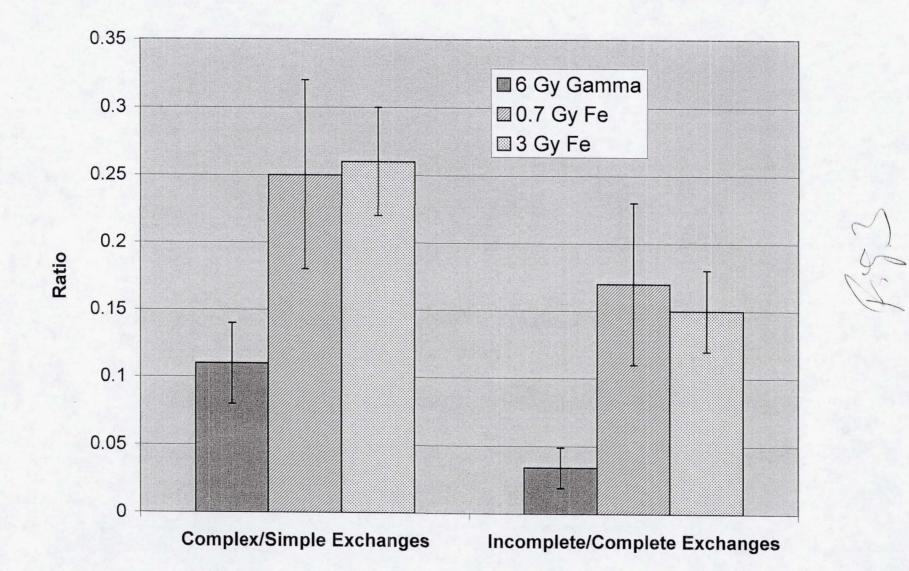
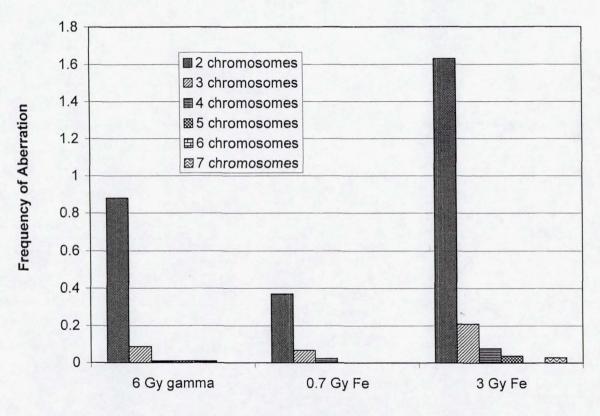| Radiation	LET (keV/μm)	Dose (Gy)	Cells scored	Complete exchange	True Incomplete exchange	Insertion	Interstitial deletion and centric ring	Terminal deletion	Ratio of unrejoined to misrejoined break ends	Ratio of incomplete to complete exchanges
Gamma	0.6	6	105	32 (0.05±0.01)	2 (0.003±0.002)	0	11 (0.017±0.005)	1 (0.002±0.002)	0.06±0.03	0.06±.0.04
Si 490 MeV/u	55	2	187	39 (0.10±0.02)	5 (0.013±0.006)	0	12 (0.032±0.009)	1 (0.003±0.003)	0.08±0.03	0.13±0.06
Fe 500 MeV/u	200	2	93	40 (0.22±0.03)	8 (0.043±0.015)	7 (0.038±0.007)	13 (0.070±0.019)	3 (0.016±0.009)	0.12±0.03	0.20±0.08
Fe 200 MeV/u	440	3	124	55 (0.15±0.02)	18 (0.048±0.011)	10 (0.027±0.009)	12 (0.032±0.009)	5 (0.013±0.006)	0.16±0.03	0.33±0.10

Table 2. Chromosome aberrations in human fibroblast cells exposed to γ and 200 MeV/nucleon Fe ions.

Radiation	Cells scored	Simple exchanges	Complex exchanges	Weighted C ratio	Complete exchanges	Incomplete exchanges	Ratio of incompletes to completes
Control	98	1	0		0	0	
6 Gy γ	128	111	12	0.11±0.03	119	4	0.034±0.015
0.7 Gy Fe	167	60	15	0.25±0.07	64	11	0.17±0.06
3 Gy Fe	104	167	43	0.26±0.04	183	27	0.15±0.03

True
incomplete

False
incomplete

Fig. 1

Fig 2