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Cell Killing and Chromatid Damage in Primary Human Bronchial Epithelial Cells Irradiated with Accelerated ^{56}Fe Ions

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We examined cell killing and chromatid damage in primary human bronchial epithelial cells irradiated with high-energy ^{56}Fe ions. Cells were irradiated with graded doses of ^{56}Fe ions (1 GeV/nucleon) accelerated with the Alternating Gradient Synchrotron at Brookhaven National Laboratory. The survival curves for cells plated 1 h after irradiation (immediate plating) showed little or no shoulder. However, the survival curves for cells plated 24 h after irradiation (delayed plating) had a small initial shoulder. The RBE for ^{56}Fe ions compared to ^{137}Cs γ rays was 1.99 for immediate plating and 2.73 for delayed plating at the D_{10}. The repair ratio (delayed plating/immediate plating) was 1.67 for ^{137}Cs γ rays and 1.22 for ^{56}Fe ions. The dose–response curves for initially measured and residual chromatid fragments detected by the Calyculin A-mediated premature chromosome condensation technique showed a linear response. The results indicated that the induction frequency for initially measured fragments was the same for ^{137}Cs γ rays and ^{56}Fe ions. On the other hand, ~85% of the fragments induced by ^{137}Cs γ rays had rejoined after 24 h of postirradiation incubation; the corresponding amount for ^{56}Fe ions was 37%. Furthermore, the frequency of chromatid exchanges induced by γ rays measured 24 h after irradiation was higher than that induced by ^{56}Fe ions. No difference in the amount of chromatid damage induced by the two types of radiations was detected when assayed 1 h after irradiation. The results suggest that high-energy ^{56}Fe ions induce a higher frequency of complex, unrepairable damage at both the cellular and chromosomal levels than ^{137}Cs γ rays in the target cells for radiation-induced lung cancers. © 2001 by Radiation Research Society

INTRODUCTION

At a time when manned space exploration is more a reality than a myth with the construction and occupation of the International Space Station now under way, the biological effects of low-flux galactic cosmic rays (GCR) on astronauts or crews of a spacecraft have become a major concern of the various space agencies. One useful approach in assessing the risk of exposure to high-*Z*, high-energy (HZE) particles is to investigate the responses of normal human cells in culture. Many such studies have reported that high-LET charged particles are more effective per unit absorbed dose in the induction of lethality, chromosomal damage and mutations than low-LET radiation. However, almost all of these studies have used normal human fibroblasts. It is important for a realistic risk assessment of the carcinogenic potential of HZE particles to examine biological effects using normal human epithelial cells, since the majority of human cancers are of epithelial cell origin. Grossi *et al.* reported that the RBE at the 10% survival level for human mammary epithelial cells irradiated with α particles (LET 137 keV/μm) relative to 80 kV X rays was around 1 (*1*). On the other hand, studies using immortalized human bronchial epithelial cells found higher RBE values in the range of 3–6 (*2*). Nevertheless, there are only a limited number of data sets available for normal human epithelial cells. Although there are many reports in the literature concerning the biological effects of heavy ions, few deal with HZE particles such as ^{56}Fe ions (*2–14*). Several studies have used comparatively lower-energy iron ions ranging from 300 to 600 MeV/nucleon (LET 173–500 keV/μm) accelerated at the BEVALAC facility of Lawrence Berkeley Laboratory (*3–9*). Tsuboi *et al.* (*6*) showed that the RBE for cell killing and mutation induction at the *HPRT* locus were 2.89 and 1.78 (LET 500 keV/μm), 3.29 and 3.13 (LET 300 keV/μm), and 3.47 and 4.26 (LET 200 keV/μm) for normal human fibroblasts. There is evidence that the ratio of the yield of large deletion mutations to that of point mutations produced by iron ions is much higher than the same ratio for spontaneous mutations (*9*). In general, these data show that the RBE values for iron ions are greater than those for low-LET radiations but smaller than that for α particles. Biological studies using comparatively higher-energy iron ions (1 GeV/nucleon, LET = 140 keV/μm) have been carried out at Brookhaven National Laboratory (BNL) (*2*, *10–14*). These studies are of interest because the relationship between RBE and LET for biological effects has been shown to peak between 100 and 200 keV/μm for compar-

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atively lighter ions. Using the premature chromosome condensation (PCC) technique, Durante *et al.* examined the kinetics of rejoining of chromatin breaks in human lymphocytes irradiated with either low-LET radiations (γ rays or protons) or ^{56}Fe ions. Their data indicated that, while rejoining was essentially terminated within 15 h after irradiation, 90% of the breaks induced by low-LET radiations rejoined, whereas after ^{56}Fe-ion irradiation only 50% rejoined (*10, 11*). Similarly, using the fluorescence *in situ* hybridization (FISH) technique, Wu *et al.* showed that the frequency of chromosome aberrations such as reciprocal exchanges, incomplete exchanges, deletions and complex exchanges induced by ^{56}Fe ions in either human lymphocytes or fibroblasts was much higher than that induced by low-LET radiation (*12*). However, the frequency of true incomplete exchanges detected by FISH with whole chromosome-specific probes, together with telomere probes was similar for ^{56}Fe ions and γ rays (*13*). These results suggested that the quantity of initially induced chromosome aberrations by higher-energy iron ions was similar to that induced by low-LET radiation. However, the quality of the damages induced was very different. There were more large-scale damages induced by iron ions compared to low-LET radiations. While the discrepancy may be due to differences in the repair rate of cells, information on the responses of cells after exposure to higher-energy iron ions is still limited.

In this study, we examined both cell killing and chromatid damage in primary human bronchial epithelial (NHBE) cells irradiated with high-energy ^{56}Fe ions. We chose the chemically induced PCC technique to detect chromatid damage instead of analyzing chromosomal damage in metaphase spreads because of the high frequency of G_2-phase prematurely condensed chromosome induced in NHBE cells. The detection of chromosomal aberrations using the PCC technique is useful for comparing the frequency of aberrations in cells irradiated with radiations of different quality because it reduces the LET-dependent confounding effects on the cell cycle and/or on interphase cell death (*15*). There is evidence to suggest that inhibitors of protein phosphatase such as fostriecin (*16*), okadaic acid (*17–21*) and Calyculin A (*21–24*) can induce PCC in various cells in culture. These results indicate that the use of these chemicals can induce PCC for the detection of radiation-induced chromatin damage in an easier, faster and more sensitive manner than methods based on fusion- and virus-mediated procedures.

MATERIALS AND METHODS

Cells

Primary normal human bronchial epithelial (NHBE) cells (cell line no. 8F0356), which were derived from a male, nonsmoking Caucasian donor, were obtained frozen in vials from Clonetics Corporation (San Diego, CA). The cells were cultured in serum-free bronchial epithelial basal medium (BEBM, Clonetics) supplemented with bovine pituitary extract (52 μg/ml), hydrocortisone (0.5 μg/ml), epidermal growth factor (0.5 ng/ml), epinephrine (0.5 μg/ml), transferrin (10 μg/ml), insulin (5 μg/ml), retinoic acid (0.1 ng/ml), triiodothyronine (6.5 ng/ml), gentamicin-1000 (50 μg/ml) and amphotericin-B (50 ng/ml) in a 5% CO_2 incubator at 37°C. Primary NHBE cells were thawed out and subcultured once in a 75-cm^2 plastic culture flask at a density of 1×10^6 per flask. The expanded stock culture was frozen in liquid nitrogen until use (passage 3). Freshly thawed cells at passage 4 were used in the studies. The generation time of the cells was around 35 h when they were cultured following the supplier's recommendation. The irradiated cell population was 1 h late log phase or stationary phase. We did not change the medium after irradiation. Although only a single strain of the NHBE cell stock was used in this study, the survival data for ^{137}Cs γ rays were comparable with published data for different strains of NHBE cells from the same commercial source (*25*).

Irradiation

NHBE cells at passage 4 were irradiated with graded doses of ^{137}Cs γ rays or with the ^{56}Fe-ion beam. Iron-56 ions were accelerated with the Alternating Gradient Synchrotron (AGS) at BNL. The energy of the ^{56}Fe-ion beam was 1 GeV/nucleon and the dose-averaged LET of the beam was estimated to be ~140 keV/μm at the sample position (*10–14*). The dose rate of ^{56}Fe ions was 0.4 to 0.8 Gy/min. For comparison, we used ^{137}Cs γ rays generated by a Gammacell 40 (Atomic Energy of Canada Ltd.). The dose rate for γ rays was 0.98 Gy/min. All of the irradiations were carried out at room temperature.

Cell Survival

Cells were held for 1 h after irradiation; then a sufficient number were plated onto 60-mm-diameter plastic dishes (Falcon 3002) to form 50–60 viable colonies to assess the effect of immediate plating. Another set of irradiated cells was incubated in a 5% CO_2 incubator at 37°C for 24 h for delayed plating. To determine cell survival as a function of delayed plating time for γ-irradiated cells, irradiated cultures were incubated for graded periods up to 24 h postirradiation. Colonies were fixed and stained with methanol and Giemsa solution after a 14-day incubation period. Colonies consisting of more than 50 cells were scored. The plating efficiencies for the NHBE cells on plastic dishes ranged from 2 to 5%.

Detection of Chromatin Damage

Cells were treated with Calyculin A (Wako Chemicals, Tokyo, Japan) at a final concentration of 50 n*M* for 30 min in a CO_2 incubator at 37°C. At 30 min after Calyculin A treatment, the G_2-phase PCC index (7%) was seven times higher than the mitotic index (1%) after a comparable treatment with Colcemid. Calyculin A was added 1 h after irradiation for immediate assay or 24 h after irradiation for delayed assay. Cells for delayed assay were kept in a CO_2 incubator at 37°C until Calyculin A was added. The PCC samples were prepared according to a conventional cytogenetic procedure. Briefly, cells were treated with a 75 m*M* KCl solution for 20 min at 37°C and fixed in 3:1 methanol:acetic acid. The cell suspension was dropped onto ethanol-cleaned slides, air-dried, and stained with a 5% Giemsa solution. Samples of 50 G_2-phase prematurely condensed chromosomes were scored under a light microscope. The yield of induced chromatid aberrations was determined as described previously by Savage (*26*). We scored chromatid breaks, isochromatid deletions, and acentric fragments as chromatid fragments and intrachanges and interchanges as chromatid exchanges. In the samples subjected to 24 h of postirradiation incubation, we defined existing chromatid breaks, isochromatid deletions, and acentric fragments as residual chromatid fragments.

RESULTS

Figure 1 shows cell survival curves for immediate plating and delayed plating after exposure of the cells to ^{137}Cs

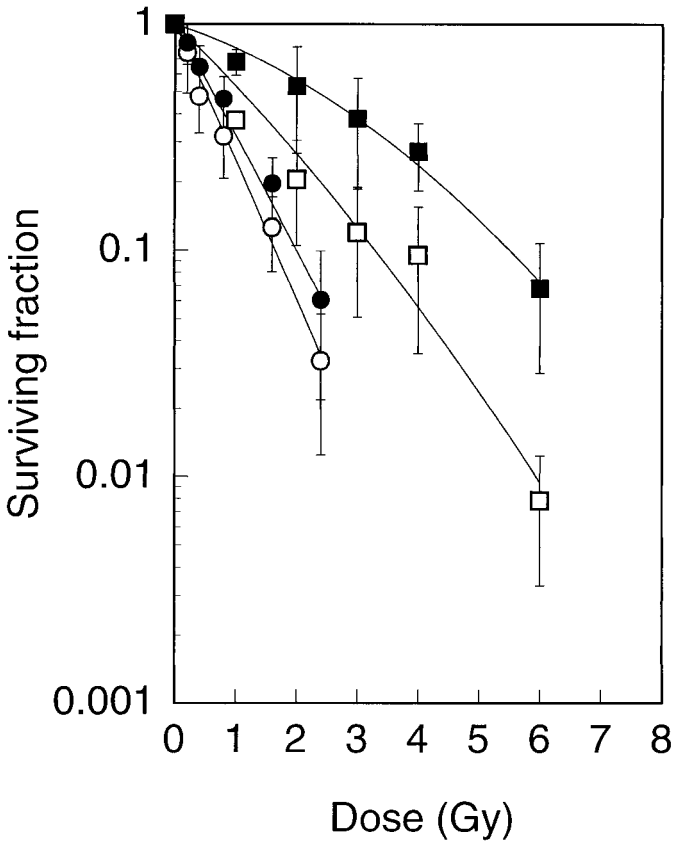

FIG. 1. Survival curves for NHBE cells irradiated with ^{137}Cs γ rays (□, immediate plating 1 h after irradiation; ■, delayed plating 24 h after irradiation) and ^{56}Fe ions (○, immediate plating; ●, delayed plating). The results are the mean and 95% confidence intervals of two independent experiments. The curves were fitted by the least-squares method to a linear-quadratic equation.

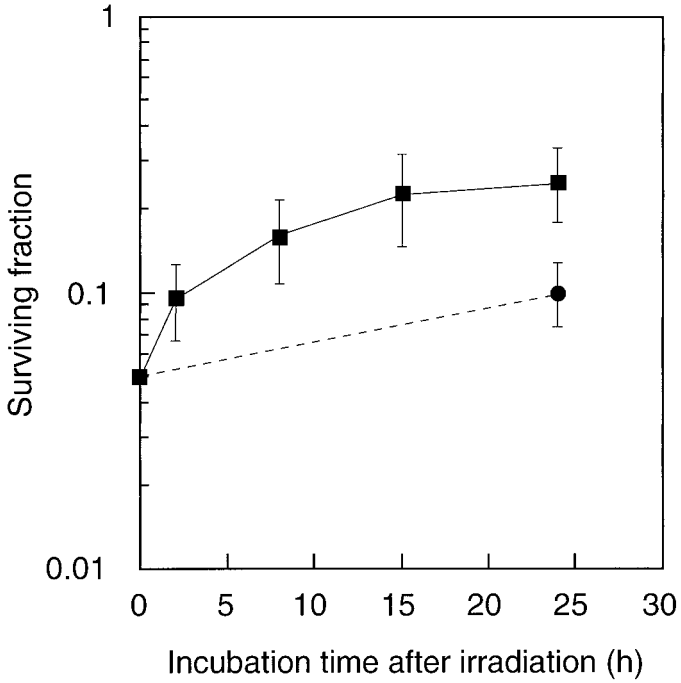

FIG. 2. Recovery time course for cell killing in NHBE cells irradiated with 4 Gy (surviving fraction ~0.05) of ^{137}Cs γ rays (■). The results are the mean and 95% confidence intervals of two independent experiments. Data for ^{56}Fe ions at 24 h (●) are taken from Fig. 1.

γ rays and ^{56}Fe-ion beams. The curves for ^{137}Cs γ rays had larger shoulders than those for ^{56}Fe ions. Similarly, the curves for delayed plating had larger shoulders than those for immediate plating for both radiation types. RBE values for ^{56}Fe ions, calculated using the D_{10}, which the dose required to reduce the surviving fraction to 10%, were 1.99 for immediate plating and 2.73 for delayed plating. The biological parameters for cell killing are summarized in Table 1. The results for ^{137}Cs γ rays were similar to those reported previously for other NHBE cell lines (*25*). We examined the time course of recovery of cells irradiated with 4 Gy of γ rays (surviving fraction for immediate plating ~0.05) (Fig. 2). Recovery was curvilinear, reaching a plateau at 15 h. This result suggested that the recovery was completed within 24 h after irradiation. It was similar to the response for repair of potentially lethal damage (PLD) in plateau-phase human osteosarcoma cells irradiated with high-LET carbon ions (*27*).

Figure 3 shows a photomicrograph of Calyculin A-mediated G_2-phase prematurely condensed chromosomes in nonirradiated (panel a) and γ-irradiated NHBE cells (panel b). In γ-irradiated cells, many chromatid-type aberrations were observed (arrows). The dose–response curves for both immediate assay and delayed assay in G_2-phase prematurely condensed chromosomes were linear over the range of

TABLE 1
Parameters for Cell Killing

Radiation and treatment	α (Gy^{-1})	β (Gy^{-2})	D_{10} (Gy)	RBE at D_{10}	σ (μm^2)[a]
^{137}Cs γ rays					
Immediate plating	0.605 (0.303–0.908)	0.0289 (0.0116–0.0468)	3.26 (2.53–3.99)		0.0314 (0.0226–0.0402)
Delayed plating	0.208 (0.085–0.332)	0.0385 (0.0390–0.0402)	5.45 (4.73–6.18)		0.0158 (0.0125–0.0191)
^{56}Fe ions (140 keV/μm)					
Immediate plating	1.38 (0.811–1.95)	0.0115 (0.0063–0.0163)	1.64 (1.31–1.97)	1.99 (1.69–2.29)	31.2 (23.3–39.1)
Delayed plating	1.12 (0.784–1.42)	0.0158 (0.0129–0.0186)	2.00 (1.73–2.27)	2.73 (2.54–2.92)	25.6 (21.6–29.6)

Note. The data are the mean and 95% confidence intervals from two independent experiments.

[a] Inactivation cross section (μm^2) = 0.16021 × LET (keV/μm)/D_{37} (Gy), where D_{37} is determined as the dose (Gy) required to reduce the surviving fraction to 37%.

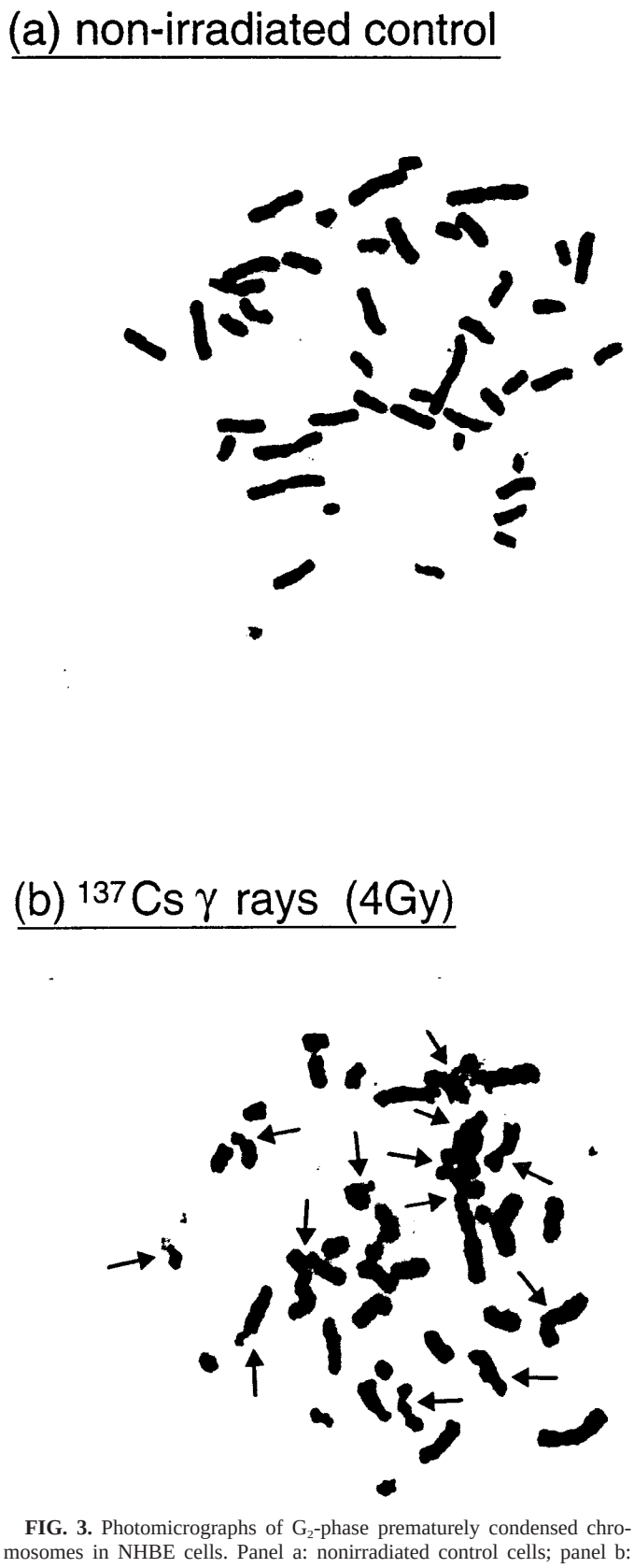

FIG. 3. Photomicrographs of G_2-phase prematurely condensed chromosomes in NHBE cells. Panel a: nonirradiated control cells; panel b: cells irradiated with 4 Gy of ^{137}Cs γ rays. Arrows show chromatid damage. Original magnification 1000×.

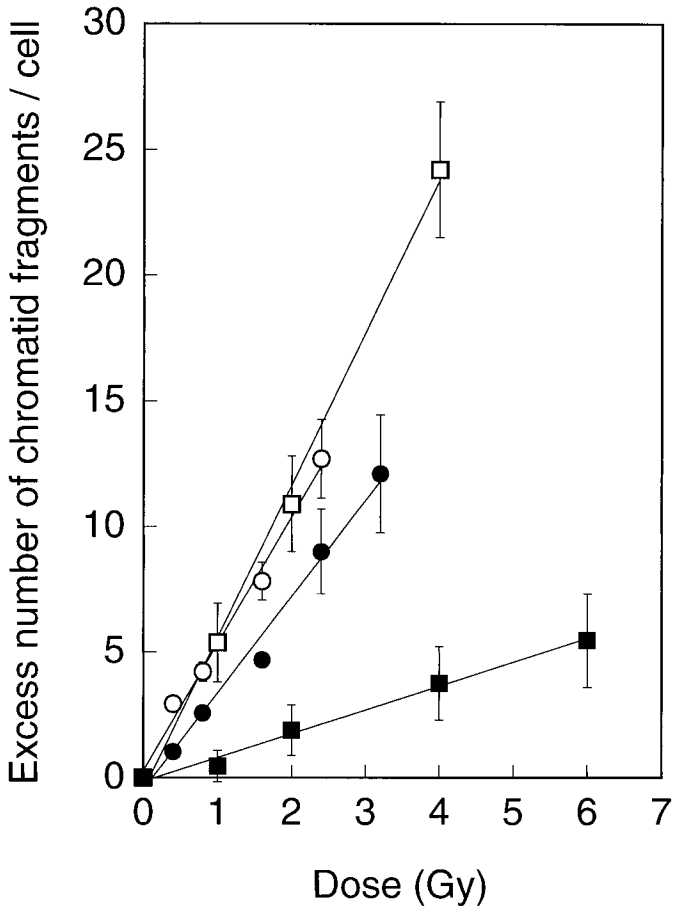

FIG. 4. Dose–response curves for chromatid fragments (chromatid breaks, isochromatid deletions and acentric fragments) in cells irradiated with ^{137}Cs γ rays (□, immediate assay 1 h after irradiation; ■, delayed assay 24 h after irradiation) and ^{56}Fe ions (○, immediate assay; ●, delayed assay). The results are the mean and 95% confidence intervals of the two independent experiments. The curves were fitted by the least-squares method.

doses examined (Fig. 4). The results indicate that the induced frequency for initially measured fragments by ^{56}Fe ions was the same as that for γ rays. The number of chromatid fragments per gray per cell was 5.56 for γ rays and 5.33 for ^{56}Fe ions. On the other hand, the frequency of residual fragments, i.e. the damage remaining after 24 h, in NHBE cells irradiated with ^{56}Fe ions was ~4.2-fold higher than that in cells irradiated with γ rays. The number of residual fragments per gray cell was 0.80 for γ rays and 3.37 for ^{56}Fe ions (Table 2). These results suggest that the damage induced by γ rays and ^{56}Fe ions was qualitatively different.

Figure 5 shows the time course of rejoining after irradiation with 4 Gy of γ rays. It was remarkable that the induced fragments rejoined quickly and gradually decreased up to 15 h before reaching a plateau. Around 85% of the fragments induced by γ rays had rejoined after 24 h of postirradiation incubation, while only 37% of the fragments induced by ^{56}Fe ions had rejoined. These data pro-

TABLE 2
Summary of Analysis of Data for Chromatid Fragments

Radiation and treatment	Residual fragments per cell per gray	RBE[a]	Percentage rejoining at 24 h
^{137}Cs γ rays			
Immediate assay	5.56 (3.81–7.31)		
Delayed assay	0.80 (0.23–1.31)		85.6 (77.0–94.2)
^{56}Fe ions (140 keV/μm)			
Immediate assay	5.33 (4.91–5.73)	0.96 (0.65–1.3)	
Delayed assay	3.37 (3.12–3.61)	4.2 (3.0–5.5)	36.8 (29.6–45.9)

Note. The data are the mean and 95% confidence intervals of two independent experiments.
[a] RBE was calculated using the slope of the dose–response curves.

vide clear evidence that ^{56}Fe ions produce non-rejoining chromatid fragments much more effectively than γ rays.

Figure 6 shows the dose–response curves for chromatid exchanges. The curves for delayed assay were curvilinear for both radiation types. The frequency for delayed assay for γ rays was higher than that for ^{56}Fe ions, but there was no difference in immediate assay between γ rays and ^{56}Fe ions. A low number of exchanges were observed in immediate assay for both radiation types. This result suggested that some rejoining occurred during the 1.5 h incubation and treatment with Calyculin A postirradiation. The dose of γ rays was required to produce 0.15 exchanges per cell 1.6 times greater than the dose of ^{56}Fe ions.

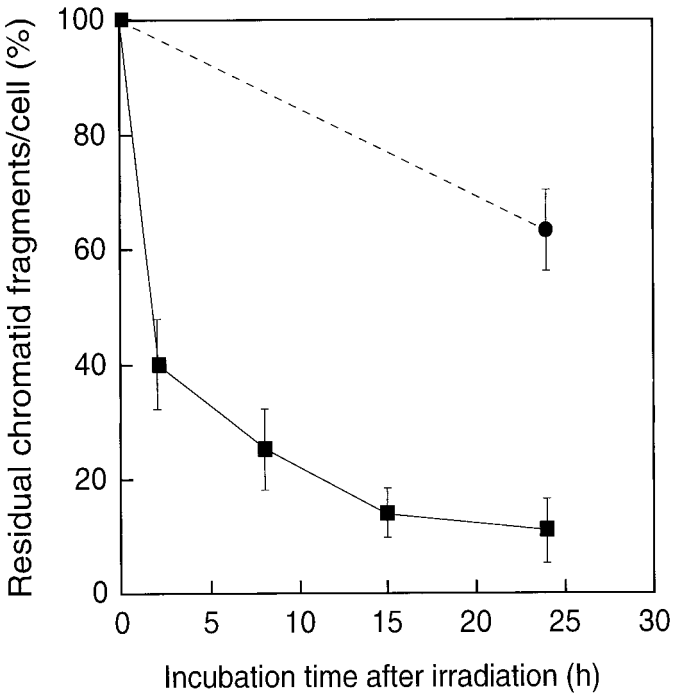

FIG. 5. Time course for rejoining of chromatid fragments (■) induced by 4 Gy of ^{137}Cs γ rays. The results are the mean and 95% confidence intervals of two independent experiments. Data for ^{56}Fe ions at 24 h (○) are taken from Fig. 4.

DISCUSSION

It has been reported that the RBE values of comparatively lower-energy iron ions (300–600 MeV/nucleon, LET = 173–500 keV/μm) for cell killing was 2.9–3.5 in normal human fibroblasts (*6*). Using different charged particles (LET ~150 keV/μm) with the exception of iron ions, Barendsen *et al.* (*28*) showed that the RBE value for cell killing in human kidney cells irradiated with α particles from ^{210}Po

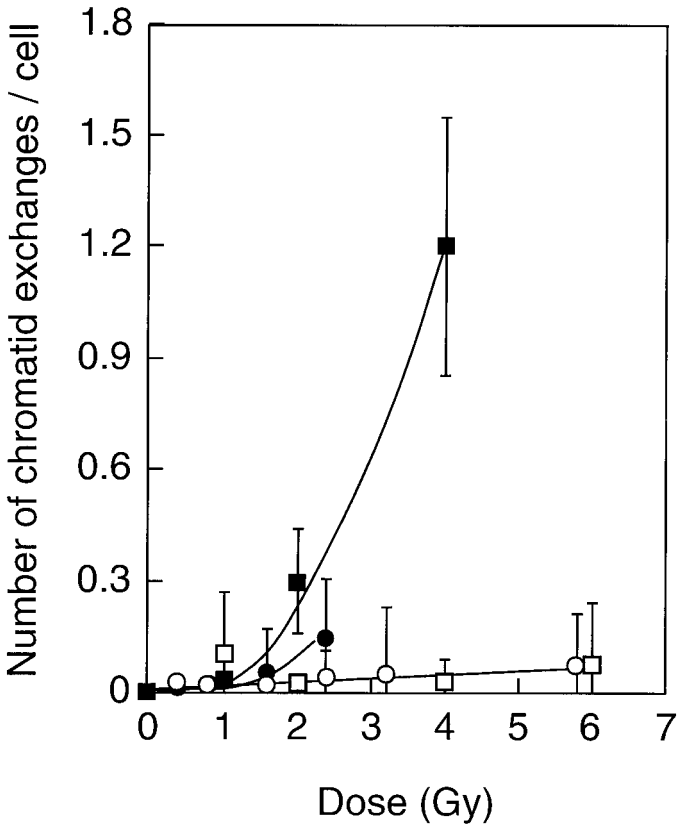

FIG. 6. Dose–response curves for chromatid exchanges (intrachanges and interchanges). (□, immediate assay 1 h after irradiation; ■, delayed assay 24 h after irradiation) and ^{56}Fe ions (○, immediate assay; ●, delayed assay). The results are the mean and 95% confidence intervals of two independent experiments. The curves were fitted by the least-squares method.

was 3.6 at the 10% survival level. Other studies showed that the RBE was 3.5 for 165 keV/μm boron ions (*29*), 2.4 for ~110 keV/μm argon ions (*30*), 2.5–3.0 for 145–162 keV/μm carbon ions and 150–l55 keV/μm neon ions (*31*), 3.31 for 105.8 keV/μm α particles (*32*), 3.19 for 131 keV/μm neon ions (*33*), and ~3.5 for 153.3 keV/μm carbon ions (*34*) at the 10% survival level using cells of either human or rodent repair-proficient cell lines. Our result (RBE = 1.99) for immediate plating is smaller than those of the above-mentioned studies. One possible explanation for this difference is that the maximum peak for RBE–LET relationship changed to higher-LET regions. According to the previous reports (*32–36*), the maximum peak in RBE moves to higher-LET regions for heavier ions. The RBE values for ^{56}Fe ions obtained in the present study are therefore smaller than that for lighter ions. Conversely, the inactivation cross section for immediate plating is 0.03 ± 14 μm^2 for γ rays and 31.2 μm^2 for ^{56}Fe ions. These values are similar to those for other *in vitro* and *in vivo* studies reported previously (*31, 34, 37*). The results suggest that the target of radiation-induced reproductive cell death for primary human bronchial epithelial cells is the same as that for other human and rodent cells.

The α/β ratio, which is an indicator of radiation repair capacity (*34, 38*), is 20.9 for immediate plating and 5.4 for delayed plating in γ rays. These values are much smaller than those of 120 for immediate plating and 70.9 for delayed plating obtained for ^{56}Fe ions. The data are consistent with the different shapes of the survival curves obtained for γ rays and ^{56}Fe ions. Furthermore, based on the repair ratio (D_{10}[delayed plating]/D_{10}[immediate plating]) of 1 .67 for γ rays and 1.22 for ^{56}Fe ions, these results indicate that HZE-particle beams are more effective for cell killing than low-LET γ rays.

There are many reports concerning the rejoining kinetics of radiation-induced chromosomal damage by PCC using either human or rodent cell systems. Hittelman and Rao (*39*) demonstrated that the frequencies of all aberration types (gaps, breaks and exchanges) in the G_2-phase prematurely condensed chromosomes induced by X rays declined significantly by 20 min after irradiation, and this decline continued even up to 45 min. Furthermore, the time course of rejoining of radiation-induced chromatin fragments in the G_1-phase prematurely condensed chromosomes reached a plateau up to 10 h after irradiation, and the rejoining half-time ranged from 30 min to 2 h. Likewise, the maximum percentage of rejoining of fragments induced by heavy ions was dependent on LET. In contrast, over 85% of the fragments induced by low-LET radiation in either human or rodent cell systems were rejoined (*39–45*). Goodwin *et al.* (*45*) demonstrated a clear LET-dependent trend in the percentage of excess fragments remaining in Chinese hamster ovary cells irradiated with helium (0.56 keV/μm), carbon (13.7 keV/μm), argon (115 keV/μm) and neon (183 keV/μm) ions. Around 90% of the fragments induced by helium ions in CHO cells were rejoined, while only 50% of neon-ion-induced fragments were rejoined. A similar LET dependence of the yields of residual chromatin breaks was observed in normal human fibroblasts using the single ion source of carbon (*46*) and neon (*33*). The results indicated that the maximum percentage of residual fragments was around 50% for both carbon (110–124 keV/μm) and neon (121 keV/μm) ions. Using comparatively heavier ions, Durante *et al.* (*10*) showed that 50% of chromatin breaks induced on chromosome 4 in human lymphocytes by 140 keV/μm iron ions had rejoined 10 h after irradiation when analyzed using the FISH technique. Although the minimum sampling time for our study is 2 h after irradiation, γ-ray-induced chromatid fragments rejoined quickly (rejoining half-time ~30 min). In addition, around 85% of fragments had rejoined 24 h after irradiation. Although these results are qualitatively similar to the previous reports, the maximum percentage rejoining (36.8%) of iron-ion-induced fragments is lower than that of comparatively lighter ions or for the lymphocyte studies mentioned above. This discrepancy is likely to be the result of a difference in the track structure of the core and penumbra of the various ion beams (*47*). Iron-ion tracks may deposit much more energy locally than lighter ions of a similar LET. Consequently, iron ions could produce more severe clustered damage at the chromatin level than lighter ions of a similar LET. Since the same ion source was used in the present study and in the lymphocyte studies of Durante *et al.*, the exact reason(s) for the discrepancy is not known. It is likely that a difference in the origin of the cells and the assay used could account for the difference in the results.

Since the frequency of initially measured fragments was the same for ^{56}Fe ions and γ rays (Fig. 4), a larger number of exchanges could be formed by γ irradiation than ^{56}Fe-ion irradiation during postirradiation incubation. Furthermore, we observed a higher frequency of isochromatid deletions without union proximal and distal in iron-ion-irradiated samples than in γ-irradiated samples (data not shown). Although it is still unclear whether the isochromatid deletions were formed by a one-hit event or by breaking two arms at two individual events, this result suggests the possibility that HZE particles can produce more damage by one-hit events. Recently, Anderson *et al.* demonstrated that complex chromosome aberrations, which are defined as three or more breaks in two or more chromosomes, were induced at a higher frequency in human lymphocytes after exposure to α particles (121 keV/μm) than after exposure to X rays using the multiplex fluorescence *in situ* hybridization (*48*). The present results also suggest that HZE particles induce complex unrejoinable damage at the chromosomal level in NHBE cells. This result is similar to the formation of ring chromosomes induced by X rays and neon ions in normal human fibroblasts (*42*).

In this study, we examined the biological effects induced at both the cellular and chromosomal levels by high-energy ^{56}Fe ions in primary human bronchial epithelial cells. Our data suggest that the HZE-particle irradiation can lead to

more residual damage at both the cellular and chromosomal levels than lighter ions with a similar LET or than low-LET γ rays. Differences in the track structure of the energy deposition of the different ion sources may play an important role in the biological effects of HZE particles. We further show the usefulness of the Calyculin-A-mediated PCC technique for detecting chromosomal damage induced by different types of radiation.

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