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No significant level of inheritable interchromosomal aberrations in the progeny of bystander primary human fibroblast after alpha particle irradiation

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Abstract

A major concern for bystander effects is the probability that normal healthy cells adjacent to the irradiated cells become genomically unstable and undergo further carcinogenesis after therapeutic irradiation or space mission where astronauts are exposed to low dose of heavy ions. Genomic instability is a hallmark of cancer cells. In the present study, two irradiation protocols were performed in order to ensure pure populations of bystander cells and the genomic instability in their progeny were investigated. After irradiation, chromosomal aberrations of cells were analyzed at designated time points using G₂ phase premature chromosome condensation (G₂-PCC) coupled with Giemsa staining and with multiplex fluorescent *in situ* hybridization (mFISH). Our Giemsa staining assay demonstrated that elevated yields of chromatid breaks were induced in the progeny of pure bystander primary fibroblasts up to 20 days after irradiation. MFISH assay showed no significant level of inheritable interchromosomal aberrations were induced in the progeny of the bystander cell groups, while the fractions of gross aberrations (chromatid breaks or chromosomal breaks) significantly increased in some bystander cell groups. These results suggest that genomic instability occurred in the progeny of the irradiation associated bystander normal fibroblasts exclude the inheritable interchromosomal aberration.

Keywords

Genomic instability; Bystander effect; Chromatid break; mFISH; alpha-particle irradiation

1. Introduction

Genomic instability is a collective term to describe the increased rate of acquisition of alterations in the genome. It includes a variety of karyotypic abnormalities (such as

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chromosomal alterations, sister chromatid exchange (SCE) and aneuploidy), alterations in DNA repair capacity, metastatic progression, micronucleus formation, gene mutations and amplifications, microsatellite instabilities, transformation, and/or delayed reproductive death. (Morgan, 2003; Little, 2003a). Exposure of cells to ionizing radiation results in a variety of directly and indirectly induced DNA lesions, including DNA base alterations, DNA-DNA and DNA-protein cross-links, and single- and double-strand breaks (Ward, 1988). If a cell survives, the initial biological response to the radiation-induced injury may influence the cell's normal differentiation and shows the characteristic of genomic instability (Limoli et al., 1998). There are numerous evidences demonstrated that exposure to ionizing radiation may induce a heritable, genomic instability that leads to a persistent increased frequency of genetic and functional changes in the progeny of irradiated cells (Morgan et al., 1996). Chromosomal instability is a direct-characterized endpoint of radiation-induced genomic instability which was believed to be a hallmark of carcinogenesis. Measurement of cytogenetic aberrations in peripheral lymphocytes is commonly used for biological dosimetry following occupational or accidental exposure to individuals or for retrospective dosimetry, and can also help to predict the risk of side effects after radiation therapy (Kadhim et al., 2010).

The another non-targeted phenomenon that appears to be of significance recently is in regard to radiation-induced bystander effects (reviewed in [Little, 2003b; Morgan et al., 2002; Hall and Hei, 2003; Hei et al., 2011]). It was first reported by Nagasawa (Nagasawa and Little, 1992) that exposure to very low doses of alpha particles initiated sister chromatid exchanges in more cells than it was estimated could have been hit by an alpha particle. Experimental evidences obtained so far have established the existence of ionizing radiation induced "bystander effects" in diverse eukaryotic cell systems. It was defined by different biological endpoints such as chromosomal aberrations, sister chromatid exchanges, mutations, apoptosis, changes in the expression of genes and proteins (Mothersill and Seymour, 2001; Zhou et al., 2000; Zhou et al., 2004; Azzam et al., 2001; Hu et al., 2006; Hu et al., 2009). It can be induced in a variety of cell types by both low LET γ -rays (Mothersill and Seymour, 2001; Hu et al., 2009) and high LET α -particles (Zhou et al., 2000; Azzam et al., 2001; Hu et al., 2006). The harmful side of the bystander response *in vivo* has been demonstrated employing the radiosensitive *Patched-1* heterozygous mouse model system (Mancuso et al., 2008). Irradiation of the head shielded *Ptch-1* mice with 3Gy of X-rays resulted in the induction of double strand breaks and apoptosis in the cerebellar region of the brain. Likewise, cranial X-ray radiation of mice resulted in the increased DNA damage, altered cellular proliferation and apoptosis in the shielded spleen (Koturbash et al., 2008). Available evidences support two modes of signal transfer from the directly irradiated cells to bystander cells: (I) gap-junction inter-cellular communication (GJIC) (Azzam et al., 2001) and (II) secretion of soluble factors from the irradiated cells into the medium (Hu et al., 2009). Inhibitors of GJIC have been shown to reduce the levels of mutations in bystander cells. Abolition of radiation induced bystander effect by pre-treatment of cells with antioxidants indicates a role of reactive oxygen and nitrogen species in triggering the bystander responses (Shao et al., 2008; Zhou et al., 2005).

These two phenomena have been linked in a few studies, in which genomic instability was proposed to be induced from bystander cells using biological endpoints such as sister chromatid exchanges (Nagasawa and Little, 1999), micronuclei formation (Prise et al., 1998; Azzam et al., 2002; Ponnaiya et al., 2004b), gene mutation (Zhou et al., 2001) and transformation (Sawant et al., 2002). Ponnaiya et al detected elevated gross chromosomal aberrations in bystander human immortalized fibroblasts (BJ1-hTERT) cultured on the upper side of Mylar film dish after the cells on the lower side of the dish were irradiated with alpha particles with a track segment setting (Ponnaiya et al., 2004a). The elevated yields chromosomal aberrations maintained over examined time up to 25 populations post-

irradiation. In therapeutic irradiation, normal primary cells adjacent to the irradiated tumor tissue and a little part of normal tissue traversed by particles or photons may become radiation-associated bystander cells. Space radiation is characterized by low dose and low dose rate of high-energy and charge (HZE) ions from galactic cosmic radiation (Cucinotta et al., 2006), which may result in the bystander effects and increase the radiation risk for the astronaut. Nonlethal/inheritable aberrations, such as translocation, insertion, are transmissible, and can be passed on to daughter cells, which definitely increase the probability of carcinogenesis. Classic examples of inherited chromosomal instability with cancer predisposition are Bloom's syndrome, ataxia telangiectasia, Werner syndrome, and Fanconi anaemia. Thus, inheritable aberrations, which could be detected by multiplex fluorescent *in situ* hybridization assay (mFISH), should be investigated in the bystander cells for the assessment and improvement of the radioprotection.

In the present study, two protocols were carried out to ensure pure populations of bystander cells and their progeny: 1) Normal human fibroblast population was cultured in double sided Mylar dishes. Cells on lower side of the dishes were irradiated with random short penetration alpha particles at doses of 0.1 and 3Gy and thus considered as hit cells; while the cells on upper side of the dishes were bystanders. This protocol was designed to investigate the induced bystander effects by the secreted signal from irradiated cells via medium transfer: 2) Using the cell-site specific Columbia University microbeam, nuclei of 20% of cells in a near-confluent population was each irradiated with 30 alpha particles, a dose of which kills the hit cells (Zhou et al., 2000). This ensures that only the adjacent non-hit bystander cells can survive after irradiation over many generations. The aim of the latter protocol is to explore the function of gap junctional intercellular communication (GJIC) in the induction of bystander effect and the genomic instability. In both cases cells were harvested at designated time points post-irradiation and chromosomal instability was analyzed using G₂ phase premature chromosome condensation (G₂-PCC) Giemsa staining and multiplex fluorescent *in situ* hybridization (mFISH).

2. Materials and Methods

Cell culture

The normal human lung fibroblasts (MRC5) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained in MEM medium as recommended containing 10% fetal bovine serum, 100UI/ml of penicillin and 100UI/ml of streptomycin at 37°C in a humidified 95% air/5% CO₂ incubator. The population doubling time of MRC5 is about 24h.

Track segment irradiation

For the track segment irradiation, cells were co-cultured on double sided Mylar dishes as described previously (Geard et al., 2002). These dishes are stainless steel rings with ports on two opposite sides of the ring and have Mylar (6μm) on both surfaces (the distance between the two Mylar surfaces is 9mm). One of the ports is plugged. Cells were seeded onto one surface through one of the ports at a concentration of 2×10^5 cells per dish (2ml medium), and allowed to attach and incubated for 24h. The dish was then turned over, and cells were seeded onto the opposite surface (at a concentration of 4×10^5 cells per dish at 2ml medium) and allowed to attach (~1h). After completely filling the dishes with medium, they were returned to the incubator for another 24h for cell growth.

Double sided Mylar dishes with approximately 60% confluent populations of MRC5 cells on both surfaces were irradiated with 0, 0.1, or 3Gy alpha-particles (6.1MeV) generated by Radiological Research Accelerator Facility (RARAF) at Columbia University. The charged particle beam through a slot-shaped aperture in approximately in 6mm×38mm length was

generated using a 4MeV Van de Graaff accelerator. The LET of charged particle is 120KeV/ μm through the cells and the particles penetrate less than 100 μm through the 9mm medium. The beam has negligible gamma and X-ray components (Ponnaiya et al., 2004a).

After irradiation, the dishes were returned to the incubator with the irradiated surface on the lower side to avoid detachment of irradiated cells from the Mylar surface to the bystander-population side. Irradiated cells and bystander cells were separated 24h after irradiation. Part of the cells was treated with Calyculin A (Biomol, USA) for 24h time point analysis. Others were trypsinized, pooled and reseeded onto T-75 flasks. At day 5, 10, 15 and 20, the progeny of irradiated and bystander cells were collected for cytogenetic analysis.

Microbeam irradiation

Approximately 2000 exponentially growing MRC5 cells in 2 μl volume were plated onto the center of a 3.8- μm -thick polypropylene film attached on the center of a 60-mm diameter non-tissue-culture dish (Zhou et al., 2001). The nuclei of cells were stained with 100nM Hoechst 33342 for 45min before microbeam irradiation (Zhou et al., 2001). 20% of the stained cells were targeted at the nuclear centroid with 30 particles. Similarly handled sham irradiated cells were used as controls. After irradiation, fresh medium was immediately added and cells were maintained overnight. On the next day, cells were trypsinized and replated into flasks. At day 5, 10, 15, 20 after irradiation part of cells were treated for cytogenetic analysis and the rest were subcultured till the designated time points.

Cytogenetic analyses by *Giemsa staining* and mFISH assay

1) Preparation of slides—The Calyculin A premature chromosome condensation (PCC) technique was used to collect the G₂-phase cells as described (Terzoudi et al., 2003). Briefly, one day before the designated time points, cells were sub-cultured overnight. Cells were then treated with Calyculin A (50nM in DMSO) for 30min in a CO₂ incubator at 37°C. After treatment with Calyculin A, cells were easily detached from culture flasks. Prematurely condensed chromosome samples were prepared according to a conventional cytogenetic procedure. Briefly, cells were treated with 75mM KCl solution for 15min at 37°C and fixed in an ice-cold 3:1 mixture of methanol:acetic acid for 30 min and repeated the fixation for 2–3 times. Cells were either processed immediately or frozen at –20°C after fixation.

2) Giemsa staining and scoring—The cell suspensions were dropped onto pre-cleaned slides, air-dried and stained with a 5% Giemsa solution. At least 150 images of G₂-phase cells in each experimental sample were captured using digital camera under a light microscope. Chromatid-type aberration was scored as reported previously by Savage (Savage, 1976).

3) mFISH hybridization—MFISH hybridization was carried out as described (Hu et al., 2012). The samples were dropped onto cleaned slides, air-dried and aged overnight before hybridization. The slides were pretreated for 90 sec with pepsin to digest cytoplasmic proteins, and then rinsed in 1 $\times$ PBS. Each slide was post-fixed for 10 min using 1% formaldehyde in 1 $\times$ PBS+50mM MgCl₂, slides were rinsed in 1 $\times$ PBS and followed by 30 min in 2 $\times$ SSC at 75°C for denaturation of chromosomes, and then cool to room temperature (RT). Following transfer of slides to 0.1 $\times$ SSC at RT for 1min, chromosomes were denatured again in 0.07N NaOH at RT for 1 min, followed by pre-cooled incubation in 0.1 $\times$ SSC and 2 $\times$ SSC at 4°C for 1 min each. The samples were dehydrated sequentially in 70% and 100% ethanol for 3 min each, and then air dried. The denatured 24XCyte mFISH probe cocktail (Metasystems, Germany) was pipetted onto the denatured chromosomes, overlaid with a coverslip, and sealed with rubber cement and incubated in a humidified chamber at 37°C for

a minimum of 2 days. Post-hybridization washed slide with 1×SSC at 75°C for 5 min and followed by washing with 4×SSCT (4×SSC containing 0.05% Tween-20) at RT for 5 min. Blocked the non-specific targets with provided milk buffer for 30 min, followed by application of biotin labeled probes (Metasystems, Germany) with Cy5 for 30 min at 37°C. Slides are washed for 2×5 min in 4×SSCT, air dried, and mounted using DAPI/antifade (Metasystems, Germany) and overlapped with 24×60 mm² coverslip. Slides were stored at 4°C or −20°C prior to analysis.

Microscope analysis was performed using a Zeiss Axioplan 2 imaging fluorescence microscope (Zeiss, Germany) with motorized stage and filter sets for FITC, Spectrum Orange, Texas Red, Cy5, DEAC and DAPI. At least 100 images except the 3Gy irradiation group were auto-captured, processed and analyzed using Isis imaging software (Metasystems, Germany).

Statistical analysis

Statistical significance between the data on chromosomal aberration frequencies in the progeny of radiation associated bystander cells and sham irradiated control group cells was determined by χ^2 -test. Differences were considered significant at least $p < 0.05$.

3. Results and discussion

Induced chromatid breaks after track segment irradiation

Cells attached to the bottom Mylar film were irradiated with 0, 0.1 and 3Gy of alpha-particles. Associated bystander populations of MRC5 cells were examined for chromosomal aberrations every 5d up to 20d post-irradiation. A total of 150 G2 phase spreads were scored. Table 1 shows the number of chromatid breaks and their frequencies in bystander populations associated to the sham-irradiated cells ranged between 0.35 and 0.51 per cell over the time points examined. Elevated yields of chromatid breaks were observed in bystander cells associated to the irradiated cells with both doses. The frequencies of chromatid breaks in bystander cell ranged from 0.47 to 0.71 after 0.1Gy and 0.54 to 0.87 after 3Gy irradiation, respectively. The minimum and maximum fold change of 0.1Gy irradiation was 1.27 (by day 5 and day 20) and 1.65 (by day 15), respectively; while that of 3Gy irradiation was 1.46 (by day 20) and 1.83 (by day 1). χ^2 -test showed statistically significant increase ($p < 0.05$) in the numbers of chromatid breaks per cell in bystander cell populations of both dosages except 0.1Gy at day 20. Figure 1 also shows a marginal increase of chromatid breaks after 3 Gy irradiation compared with that of 0.1Gy irradiation, which is similar to the results found by Buonanno et al. (2011) that the higher dose of high-LET heavy ion radiation induced more bystander killing. Within the track segment irradiation system, there was no direct contact between the irradiated cells (bottom side) and the associated bystander cells (upper side). These results suggest that bystander cell responses and subsequent genomic instability were induced via the bystander signal molecules secreted to the medium from the irradiated cells.

To compare the relative chromatid break level between irradiated cells and bystander cells, we scored the damage in cells that attached to the bottom Mylar film and thus directly irradiated by 0.1Gy alpha particles. G2-phases of the population exposed to 3Gy were not scored because very few cells survived the irradiation and there was extremely high frequency of chromosome aberrations (data not shown). Last row of table 1 shows the numbers and the fraction of chromatid breaks per cell observed in MRC5 cells exposed to 0 and 0.1Gy alpha particles post-irradiation. The minimum fold change was 1.33 (by day 10) and the maximum fold increase was 2.27 (by day 5) after irradiation. χ^2 -test showed statistically significant aberration increases in directly irradiated cell population at each examined time point compared to the control. Chromatid breaks in cells irradiated with

0.1Gy was comparable to those observed in bystander cells. At certain time points, the frequency was even lower than those induced in bystander cells. In current study, the background of the chromatid break in control cells is higher. It may be caused by the treatment of Calyculin A. Premature condensation using Calyculin A induced breaks at the interface of early and late replicating chromosome bands bearing common fragile sites (El Achkar et al., 2005).

Induced chromatid breaks after microbeam irradiation

Randomly, nuclei of 20% cells of near-confluent fibroblasts were irradiated with 30 alpha particles using the cell-site specific precise Columbia University microbeam. The dose of 30 alpha particles, which is equal to a dose of 3Gy, lead to 100% cell death for target cells after irradiation (Zhou et al., 2000). The pure bystander cells and their progeny were therefore ensured. Table 2 shows the induction of chromatid break in the progeny of bystander cells as a function of time after irradiation. The observed chromatid breaks per cell ranged from 0.57 to 0.8 over examined time points. There was a range from 0.42 to 0.54 in the control cells. The minimum and maximum fold increase of chromatid break was 1.27 by day 15 and 1.48 by day 10, respectively. The significant differences tested by χ^2 persisted over examined time points between the bystander and control groups. These results show chromosomal instability occurred in the progeny of bystander cells.

The yields of chromatid breaks induced in the progeny of the bystander cells after both track segment and microbeam irradiation were fluctuant with the time in culture (Fig. 1). It is consistent with the observation of Ponniaya et al.(2004a), which reflects the feature of the relative fluctuation of the radiation-induced genomic instability.

Induced chromosomal aberration detected by multiplex FISH hybridization

As multiplex fluorescence in situ hybridization (mFISH) allows unambiguous identification of every human chromosome, this technique was widely utilized to precisely identify the unstable chromosomes involved in aberration formation such as breaks (chromosome and chromatid types), fragments (centric and acentric), translocations (reciprocal and nonreciprocal) and fusion in irradiated cells. Here mFISH assay was carried out for irradiated and bystander cells at 24h to 5d after track segment irradiation as well as 5 and 10d after microbeam irradiation. Supplementary figure 1 is representative picture showing some specific chromosomal aberrations induced in irradiated cells 24h after 3Gy irradiation with track segment system.

Table 3, 4 and 5 lists chromosomal aberration types and number observed in directly irradiated and associated bystander cells after irradiation with different systems. As shown in Table 3, in terms of total number of aberration scored, there was no significant difference either in directly irradiated or bystander cells 24h after exposed to 0.1Gy alpha particles. However, significant increase of gross aberrations was observed in irradiated and bystander cells 24h after 3Gy irradiation (Table 3). A total of 174 aberrations was observed in 24 G2-phase cells who survived after 3Gy track segment irradiation ($p<0.01$). In bystander cells associated to 3Gy exposure, a total of 63 aberrations was found from 133 G2-phase spreads ($p<0.01$). Meanwhile, chromatid breaks in bystander cells associated to 3Gy exposure was significantly higher than control.

Table 4 shows the induced aberration by day 5 after track segment irradiation. A total of 45 aberrations were observed in 153 G2-phase cells which are the progeny of cells exposed to 0.1Gy alpha particles, which was statistically significant when compared to control ($p<0.01$). Total numbers of aberrations in the progeny of both directly exposed to 3Gy irradiation and bystander cells remained significantly higher over the control till day 5.

Table 5 shows the chromosomal aberrations by mFISH scoring in the progeny of cells after microbeam irradiation, among which nuclei of 20% cells were irradiated with 30 alpha particles. Here only difference of chromosome break between irradiated and control cells at day 5 was statistical significant. These results may also suggest that mFISH assay is not a good tool to measure the gross chromosomal aberrations occurred in bystander primary fibroblasts, compared to the Giemsa staining assay.

Multi-FISH technique is a good approach to identify subtle change of chromosome and inheritable chromosomal aberrations, e.g., reciprocal or non-reciprocal translocation and insertion, which are stable within the cells and was detected several decades after exposure (Marder and Morgan, 1993). Considerable chromosome breaks and translocations were observed in survived cells exposed to 3Gy alpha particles (track segment irradiation). The track segment irradiation is a broad spectrum irradiation which fit the Poisson distribution. The deposited dose in nuclei for a little part of irradiated cells will be less than 3Gy so that we can get a little survived cell. Some inheritable chromosome aberrations were also observed in directly irradiated cells by 0.1Gy alpha particles. Although some cases of chromosomal interchange (translocation) were measured in the bystander cell groups, there were no significant level of inheritable interchromosomal aberrations occurred in bystander cells as shown in Table 3, 4 and 5, compared to the control. Ponnaiya et al. found unstable single cell clones with de novo translocation isolated from nonirradiated bystander human bronchial epithelial (HBEC-3kt) cells received the medium from silicon-ion irradiated cultures (Ponnaiya et al., 2011). Our previously study also measured the intrachromosomal instability clone isolated from the bystander A_L cells (Hu et al., 2012). However, the cells used in both two studies were the immortal cell lines. It was argued by Dugan et al. that starting with cells that are tumor-derived or immortalized or are in some way abnormal in the processing of radiation damage may already provide the kind of precondition that may be necessary for radiation-induced instability (Dugan and Bedford, 2003).

Cellular irradiation leads to the production of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxy radicals, which can indiscriminately damage a host of cellular components (Morgan et al., 2002; Morgan, 2002). Studies have shown that bystander effect could be induced via cyclooxygenase-2 (COX-2) pathway (Zhou et al., 2005) and reactive oxide species played a role in radiation-induced bystander effect (Azzam et al., 2002; Shao et al., 2008). It was also suggested that ROS contribute to the induction of sister chromatid exchanges (SCEs) in bystander cells (Deshpande et al., 1996; Lehnert et al., 1997; Narayanan et al., 1997). Cells exposed to the densely ionizing radiations induce heavily clustered DNA damages that are difficult to repair correctly. Damage clustering is reflected in the complexity of mutations and intra- and interchromosomal insertions and inversions occur at the sites of some large deletions (Singleton et al., 2002). It is possible that most of the DNA damages induced by ROS/RNS or other signaling molecules secreted from irradiated cells are sparsely and simple chromosomal aberrations are observed in bystander cells. Cells with chromosomal damage experience DNA repair or apoptotic or necrotic processes. Continuous free radicals derived from the fraction of the dead cells increase the level of chromatid aberration in the next generation of the cells and display genomic instability in both the bystander and irradiated cells (Limoli et al., 1998).

4. Conclusions

Our data showed that the frequencies of chromatid break in bystander cells significantly increased and persisted up to the indicated time after both track segment and microbeam irradiation. There was no significant level of inheritable interchromosomal aberrations in the progeny of the irradiation associated bystander normal fibroblasts in the duration of culture

in our current study. These results suggest that genomic instability occurred in the progeny of the bystander fibroblasts exclude the inheritable interchromosomal aberration. Several studies have showed ROS/RNS contributed the induction of radiation induced bystander effect (Hu et al., 2006; Azzam et al., 2002; Shao et al., 2008; Deshpande et al., 1996; Lehnert et al., 1997; Narayanan et al., 1997). Taken together, it may suggest that the administration of some anti-free radical drugs may provide some efficient protections of the radiation-induced bystander effect and which may initiate the carcinogenesis process during and after radiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- Gross chromosomal aberrations are higher in the progeny of the bystander normal fibroblasts.
- No significant level of induced inheritable interchromosomal aberrations occurred in the progeny of the bystander fibroblasts.
- Suggestion that administration of some anti-free radical drugs may provide some efficient protections of the radiation-induced bystander effect during and after radiation.

Figure 1.

Comparisons among the induced chromatid breaks in the irradiated and bystander cells as a function of time after both track segment and microbeam irradiation. Induced chromatid breaks per cell fraction = chromatid breaks incidence minus background in the corresponding control.

Chromatid breaks in bystander and irradiated cells after track segment irradiation. 0.1Gy-By and 3Gy-By represents the samples from the bystander cells associated to the irradiated cells by 0.1Gy and 3Gy alpha particles, respectively. 0.1Gy-Di represents the samples from the directly irradiated cells by 0.1Gy alpha particles.

Table 1

Group	24 h after Irradiation		5 d after Irradiation		10 d after Irradiation		15 d after Irradiation		20 d after Irradiation	
	Control	Irradiation	Control	Irradiation	Control	Irradiation	Control	Irradiation	Control	Irradiation
10 cGy-By	No. of scored spreads		150	150	150	150	150	150	150	150
	Total No. of Chromatid breaks		52	86**	76	98*	71	93*	65	107**
	Aberrations per cell		0.35	0.57	0.51	0.65	0.47	0.62	0.43	0.71
3 Gy-By	No. of scored spreads		150	150	150	150	150	150	150	150
	Total No. of Chromatid breaks		52	96**	76	130**	71	115**	65	117**
	Aberrations per cell		0.35	0.64	0.51	0.87	0.47	0.77	0.43	0.78
10 cGy-Di	No. of scored spreads		150	150	150	150	150	150	150	150
	Total No. of Chromatid breaks		51	77**	55	126**	65	85*	69	99**
	Aberrations per cell		0.34	0.51	0.37	0.84	0.43	0.57	0.46	0.66

* $p<0.05$,
** $p<0.01$.

Chromatid breaks in the progeny of cells where nuclei of 20% of cell were irradiated with 30 alpha particles using microbeam facility.

Table 2

Group	5 d after Irradiation		10 d after Irradiation		15 d after Irradiation		20 d after Irradiation	
	Control	Irradiation	Control	Irradiation	Control	Irradiation	Control	Irradiation
No. of scored spreads	150	150	150	150	150	150	150	150
Total No. of Chromatid breaks	63	87**	81	120**	67	85*	70	97**
Aberrations per cell	0.42	0.58	0.54	0.8	0.45	0.57	0.47	0.65

* $p<0.05$,
** $p<0.01$.

Multiplex-FISH scoring of chromosomal aberrations in irradiated and the associated bystander cells 24h after track segment irradiation. 0.1Gy-Di and 3Gy-Di represents the directly irradiated cells by 0.1Gy and 3Gy alpha particles, respectively. 0.1Gy-By and 3Gy-By represents the bystander cells associated to the irradiated cells by 0.1Gy and 3Gy alpha particles, respectively.

Table 3

	Control	10cGy-Di	10cGy-By	3Gy-Di	3Gy-By
No. of scored spreads	127	143	140	24	133
Chromatid breaks	25	21	40	10 [*]	51 ^{**}
Chromosome breaks	13	21	7	135 ^{**}	11
Chromosomal interchanges	1	3	2	29 ^{**}	1
Total No. of aberrations	39	45	49	174 ^{**}	63 ^{**}
Total No. of aberrations per cell	0.31	0.31	0.35	7.25	0.47

^{*} $p<0.05$,

^{**} $p<0.01$.

Table 4

Multiplex-FISH scoring of chromosomal aberrations in irradiated and the associated bystander cells 5d after track segment irradiation. 0.1Gy-Di and 3Gy-Di represents the progeny of the directly irradiated cells by 0.1Gy and 3Gy alpha particles, respectively. 0.1Gy-By and 3Gy-By represents the progeny of the bystander cells associated to the irradiated cells by 0.1Gy and 3Gy alpha particles, respectively.

	Control	10cGy-Di	10cGy-By	3Gy-Di	3Gy-By
No. of scored spreads	141	153	154	14	155
Chromatid breaks	14	33**	15	0	38*
Chromosome breaks	10	11	15	139**	6
Chromosomal interchanges	0	1	2	43**	1
Total No. of aberrations	24	45*	32	182**	45*
Total No. of aberrations per cell	0.17	0.29	0.21	13.00	0.29

* $p<0.05$,
** $p<0.01$.

Table 5

Multiplex-FISH scoring of chromosomal aberrations in the progeny of bystander cells 5d and 10d after microbeam irradiation.

Group	5d after Irradiation		10d after Irradiation	
	Control	Irradiation	Control	Irradiation
No. of scored spreads	135	141	121	142
Chromatid breaks	29	33	14	23
Chromosome breaks	8	19 [*]	7	12
Chromosomal interchanges	4	4	2	4
Total No. of aberrations	41	56	23	39
Total No. of aberrations per cell	0.30	0.40	0.19	0.27

^{*}
 $p < 0.05$.