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Ring chromosome aberrations in human lymphocytes induced by 5–20 Gy carbon ion irradiation

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Abstract In this paper, we study the biological response induced by heavy ions irradiation of high dose, human peripheral blood lymphocytes are irradiated *in vitro* by the carbon ions of LET=35 keV/μm, and the chromosome aberrations at absorbed doses of 0–20 Gy are analyzed by the calyculin A-induced premature chromosome condensation (PCC). The frequencies of PCC-rings at the stage of G2/M-phase increase steeply with radiation doses up to 20 Gy at a rate of 0.017 Gy^{-1}. The G2-PCC index remains more than 5% up to 15 Gy, and 3% after 20 Gy, this is high enough to score a substantial number of chromosome spreads without dose-rate effect. The results show that the calyculin A-induced PCC technique is suitable for analyzing the chromosome damage induced by carbon ions irradiation of high dose.

Key words Carbon ion beams, Human lymphocytes, Prematurely condensed ring chromosome.

1 Introduction

Heavy ions are characterized by their high linear energy transfer (LET) and the Bragg peak at the end of their tracks, hence increased biological effectiveness in the peak region, and ^{12}C ion beams are attractive in radiotherapy[1,2]. Chromosomal aberration, a sensitive indicator of radiation-induced genetic alterations, has been extensively studied in the dose range of radiotherapy using carbon ion beams[3,4]. The method to prepare chromosomes by the colcemid block protocol is well established. However, doses over 10 Gy cannot be measured accurately by the dicentric assay. PBLs remaining in the peripheral blood arrest at G2 or G1 phases, and do not enter mitosis, thus it is impossible to obtain mitotic chromosomes from the PBLs. Also, the dicentrics production saturates at high doses, and insensitive dose response will yield little information about radiobiological effects.

For investigating radiobiological effects at high doses, premature chromosome condensation (PCC) technique is used to analyze cell damage in condensed interphase chromosomes and overcome radiation-induced cell cycle arrest with low mitotic index[5–10]. PCC can be induced by lymphocytes fusion with mitotic cells[5], but a better way is to induce PCC by calyculin A or okadaic acid at any phase of the cell cycle[11]. Using Calyculin A-induced PCC technique, Gotoh *et al.*[12–14] studied the chromosome fragment aberrations in cells irradiated with 40 Gy ^{60}Co γ-rays; and Kanda *et al.*[15,16] and Lamadrid *et al.*[17] studied the chromosome ring aberrations in cells irradiated to ~20 Gy by X-rays or 10 Gy by neutrons. For irradiation of heavy ions, however, the dose has not exceeded 10 Gy in previous studies[18–20].

In this work, the calyculin A-induced PCC technique was applied *in vitro* to carbon ion irradiation of up to 20 Gy. The dose response relationship was established by prematurely condensed chromosome

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rings at the stage of G2/M-phase[G2/M-PCC-Rs (rings)] in normal human lymphocytes, and the dose rate effect was studied.

2 Material and methods

2.1 Blood collection

Human peripheral blood from three healthy donors without exposure to ionizing radiation or clastogenic chemicals was heparinized with 100U heparin to prevent coagulation.

2.2 *In vitro* irradiation

The blood samples were irradiated by ^{12}C ion beams of 100.55 MeV/u from the Heavy Ion Research Facility in Lanzhou (HIRFL) at the Institute of Modern Physics, Chinese Academy of Sciences. They were contained in Petri dishes placed on a stage, rotatable with remote control, under the therapy terminal (a vertical beam line), and irradiated at room temperature to different doses of up to 20 Gy at about 5 Gy/min of the dose rate. The 100.55 MeV/u ^{12}C ions lose their energy in the vacuum window, the air gap, the cover of Petri dish, and the medium, hitting the blood samples with the practical energy of 69 MeV/u, which corresponds to a mean LET of 35 keV/μm. To study the dose rate effect, the samples were irradiated to 10 Gy at dose rate of about 1, 5 or 10 Gy/min.

2.3 Lymphocyte and its chromosome

The PBLs were separated from the irradiated blood using lymphocyte separation medium (Sigma). The 0.5 mL PBLs was cultured in 5.0-mL RPMI 1640 medium, supplemented with 20% fetal calf serum, 0.5-mg streptomycin, 500 IU penicillin, 500 IU heparinsodium, and 2% phytohaemagglutinin-P (PHA-P, Sigma) under 5% CO_2 and 95% humidified atmospheres at 37°C for 48 h. The Calyculin A (Wako Chemicals, Japan) dissolving in anhydrous ethanol was prepared into a 100 μM stock solution and stored at –20°C, and diluted into 100 nM by the medium to induce PCC. After 1 h, the cells were harvested, swelled in 75 mM KCl at 37°C for 20 min, and fixed with methanol:glacial acetic acid (3:1 v/v). The cells were washed and fixed three times in the same fixative, dropped onto an ice-cold glass slide and air-dried. The slides were stained with 5% Giemsa solution in 1/15 M phosphate buffered saline (PBS, pH 6.8).

2.4 Scoring criteria and statistics

Giemsa-stained PCC spreads were observed by oil immersion under an optic microscope of 1000 × magnification. At G2- and M-phase, the incidence of PCC rings was scored with more than 46 elements. Both a ring with or without a visible centromere and a couple of rings separated or united by the centromere were considered to represent a single ring[15,16]. About 200 cells of G2/M-PCC were scored at per irradiation dose, and the frequency of G2/M-PCC-Rs was the ratio of the total number of rings to the total number of observed cells. The G2-PCC index was obtained by at least 500 spreads[11].

When a Poisson distribution of aberrations in the cells was proven to be corrective and the dose was homogeneous, the confident interval of frequency could be calculated. The u-test was used to test whether the dispersion of these aberrations could be described by a Poisson distribution[21]. According to the Poisson statistical law, only 5% likelihood of the |u| value for a distribution will exceed |1.96| by chance. Another indicator of the dispersion is the variance to mean ratio (σ/Y). Statistical analyses were conducted by student *t*-test in experiment of the dose-rate effect.

3 Results

The scoring criteria of G2/M-PCC-Rs were shown in Fig.1, and the G2/M-PCC-Rs in G2/M-PCC cells at each dose were counted. Fig.2 shows that the frequency of G2/M-PCC-Rs per cell (left, y-axis) increases linearly with the dose at slope of 0.017 Gy^{-1}, and the G2/M-PCC index (right, y-axis) is more than 5 % up to 15 Gy, and around 3 % at 20 Gy.

Fig.3 shows frequency of PCC-Rs per cell induced by 10 Gy irradiation of ^{12}C ion beam at different dose-rates. The dose-rate effect is insignificant from the student t-test. Judging from the |u| value and the σ/Y ratio in Table 1, the chromosome ring distribution of an over dispersion at lower doses becomes Poisson distribution at 20 kGy.

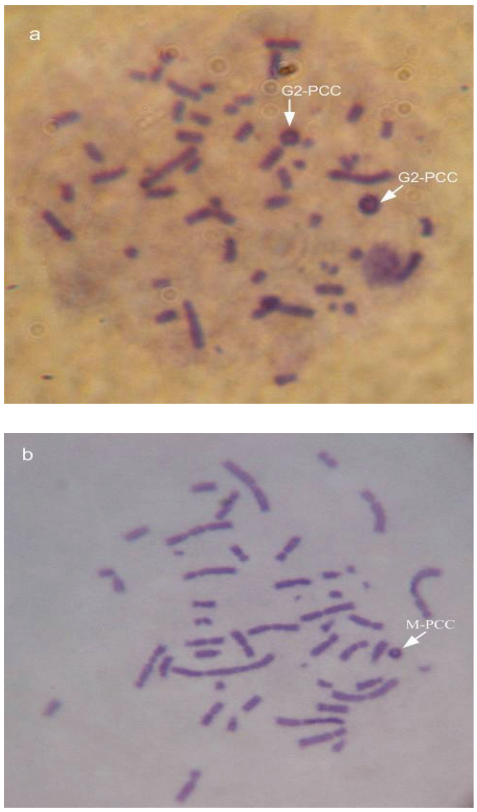

Fig.1 Calyculin A-induced and Giemsa-stained PCCs of PHA -P-stimulated PBL, 48 h after ^{12}C ion irradiation (1000× magnification). (a)G2-PCC with two rings, by 10 Gy irradiation, (b) M- PCC with one ring, by 5 Gy irradiation.

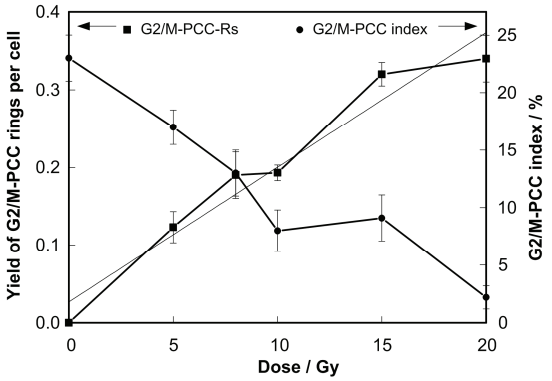

Fig.2 Dose–response to G2/M-PCC-Rs and the G2/M-PCC index.

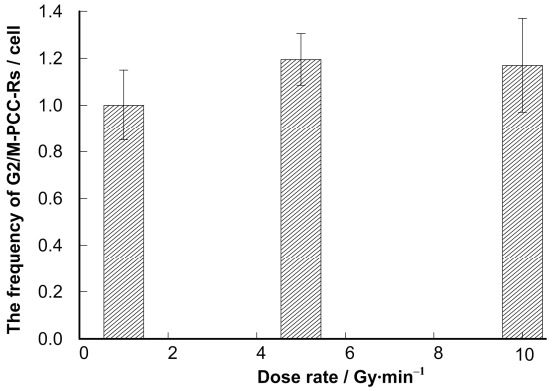

Fig.3 Frequency of PCC-Rs per cell induced by 10 Gy irradiation of ^{12}C ion beams at different dose-rates.

Table 1 Number of G2/M-PCC cells, total G2/M-PCC-Rs, and the G2/M-PCC-Rs per cell in human peripheral blood lymphocytes at different doses. The σ^2/Y and |u| values indicate that the chromosomal aberration distribution became a Poisson distribution finally.

Dose (Gy)	G2 / M-PCC cells scored	Total G2 / M-PCC-Rs	G2 / M-PCC-Rs (cell)	Distribution 0	1	2	3	4	5	σ^2/Y	|u|
0	1000	0	0	1000	0	0	0	0	0	–	–
5	415	51	0.123	373	33	9	0	0	0	1.23	3.34
8	400	76	0.190	339	47	13	1	0	0	1.23	3.27
10	336	65	0.193	285	39	10	2	0	0	1.21	2.78
15	225	72	0.320	170	40	13	2	0	0	1.21	2.24
20	216	73	0.340	159	43	12	2	0	0	1.16	1.67

A u-test at |u| <1.96 and $\sigma^2/Y \approx 1$ means a Poisson distribution of the chromosomal aberrations; while |u| > 1.96 denies the Poisson distribution, $\sigma^2/Y < 1$ means the distribution of under dispersion, and $\sigma^2/Y > 1$ means the distribution of over dispersion.

4 Discussion

Induced by 100 nM calyculin A, the G2/M-PCC index decreased with increasing doses, being 16.9 % at 5 Gy and 3 % at 20 Gy (Fig.2), which were enough to prepare chromosomes for scoring aberrations. At the slope of 0.017 Gy^{-1}, the number of G2/M-PCC-Rs was linearly correlated with the dose, with its frequency being lower than expected (Fig.2). Fig.1 shows that the number of Giemsa-stained G2/M-PCCs could be easily obtained without any special technique and instrumentation. However, the ^{12}C ion-induced chromosome aberrations shall be studied at the high doses.

Another factor affecting the frequency of G2/M-PCC-Rs is the calyculin A concentration. Kanda *et al.*[15] reported that the number of PCC cells increased with the concentration of calyculin A. The

decreased PCC-Rs yield in this work, with the same calyculin A concentration (100 nM) as in Ref.[15], is probably because that the over-treatment resulted in diffusion and short chromosomes, and this made it difficult to analyze the chromosome. In addition, the condition of hypotonic treatment and air drying may influence the results. The less spreading conditions made it difficult, too, to analyze the small rings, and reduced the frequency of total PCC-Rs.

The main purpose of the distribution study is to evaluate dose heterogeneity in cells. If the irradiation is homogeneous, a Poisson distribution of aberrations in cells is expected, and the distribution of aberrations in cells can be used to evaluate study the individuals irradiated partially in small volumes areas of the body. Our study indicate that the distribution of chromosome rings induced by carbon ions shifted from an over dispersion to a Poisson distribution at 20 Gy. This means that the high dose high-LET irradiation is homogeneous.

Dose rate effect of ionizing radiations on biological systems has been reported controversially. Many groups reported that biological effects of radiations increases with the dose rate, while others show the opposite, with increased biological effects of radiation at low dose rate[22]. In the present paper, however, we found no dose rate effect of the carbon ions on PCC-Rs in the dose rate range of 1−10 Gy/min.

5 Conclusions

The G2/M-PCC index is high enough to prepare chromosomes for scoring aberration, and the frequency of G2/M-PCC-Rs increases linearly from 0 to 20 Gy at dose slope of 0.017 Gy^{-1} without dose rate effect. The calyculin A-induced PCC technique is suitable for high dose irradiation of carbon ion beams. It can overcome two major limits of the conventional technique at high doses, i.e. the saturation of dicentrics yield and the low mitotic index. Apart from the heavy ion facility, this technique requires no particular equipment or exceptional skill, and can be performed in a short time.

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