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Relationship between cellular radio-sensitivity and naked DNA damage in mammalian cells exposed to heavy ions

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Abstract The relationship between deoxyribonucleic acid (DNA) damage and the cell death induced by ^{12}C ions irradiation was examined in four kinds of cells, Melanoma B16, cervical squamous carcinoma HeLa, Chinese hamster V79 and hepatoma SMMC-7721. Cell survival was determined by a colonogenic assay, and the sensitivity was described by D_{50} (the dose of radiation necessary to reduce the survival to 50%). For all cell lines, D_{50} ranged from 0.74 Gy to 3.85 Gy, among them B16 was the most radiosensitive to ^{12}C ions, and V79 and HeLa cells had almost the same radio-sensitivity, SMMC-7721 was the last. The induction of deproteinized DNA double-strand breaks induced by ^{12}C ions were measured by pulsed-field gel electrophoresis (PFGE), and the initial yield of the deproteinized DNA dsbs per unit dose was expressed as the DNA double break level (L). A linear dose-response curve was seen for initial DNA dsbs for all cell lines (slopes range from 0.40-0.98 (DSBs/100Mbp/Gy)). V79 was the steepest, B16 was the last. There was an inverse relationship between the initial DNA dsb and D_{50} if the B16 cell line was not considered, but there was no relativity even excludes the B16 cell line. The present results indicate that there is no relationship between cellular sensitivity and initial DNA dsb, even exclude the effects of chromatin structure.

Key words Heavy ion, Mammalian cell, Radio-sensitivity, DNA damage

1 Introduction

The development of a reliable test to assess the individual response to radiation treatment, if we can, will enable us to tailor radiation treatment planning of the patients, with increased dose for "radio-resistant", and decreased dose for "radiosensitive", hence the reduction of common tissue complications, or even suggesting alternative treatments.

Reproductive death measured by a clonogenic assay is significantly associated with treatment outcome after radiotherapy[1]. However, it takes several weeks to complete a colony formation and the cells must be proliferated *in vitro*[2], hence the unlikeliness of its use as routine predictive assay. And assays that give a direct or indirect measure of cell killing should be developed for this purpose of applications.

It is suggested that DNA double strand breaks (dsb) may lead directly to chromosome aberration and the loss of genetic material, and it is considered that dsbs are closely related to cell death. The correlation between radio-sensitivity and DNA damage in many tumor cell lines has been studied. Some studies indicated a correlation between the initial induction of DNA dsbs with cellular radio-sensitivity in mammalian cells[3,4], while others found no correlation at all between initial dsbs and radio-sensitivity[5,6]. A strong correlation between residual dsbs (after repair) and cellular radio- sensitivity for a variety of cell lines has been found in Refs.[7,8], whereas Olive P L, *et al*[9] found little correlation between radio-sensitivity and the residual DNA dsbs in human tumor cell lines, and Marples B, *et al*[10] found that the ratio of the initial/ residual DNA dsbs was correlated with cellular radio-sensitivity. After all, it is worthwhile to investigate the different relationships between cellular sensitivity and DNA dsb induction.

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Differences in chromatin structure may be related to the different results. Chapman J D, *et al*[11] found that radiation hypersensitivity was well correlated with chromatin condensation in synchronized M or G1 mammalian cells. Then they designedly investigated whether the compacted chromatin in interphase is the target that determines the widely variable surviving fractions of 2-Gy irradiated human tumor cell lines, and found that cellular radio-sensitivity was well associated with the chromatin compacted degree[12]. Stobbe C C, *et al*[13] suggested that the compaction/ dispersion status of DNA in the cell cycle might be an important factor for determining intrinsic cell radio-sensitivity.

Due to the possible influence of chromatin structure on radio-sensitivity[11-16], in this study, we used ^{12}C ions to irradiate the deproteinized DNA of four kinds of cell lines and measured the initial DNA damage using inverse pulsed-field gel electrophoresis (PFGE) to evaluate the potential of the deproteinized DNA dsbs as a predictor of cellular radio-sensitivity.

2 Materials and methods

2.1 Cell culture

Melanoma B16, cervical squamous carcinoma HeLa, and Chinese hamster V79 were provided by the Cancer Institute, Chinese Academy of Medical Sciences; hepatoma SMMC-7721 was provided by No.2 Military Medical University. All cell lines were grown in RMPI-1640 medium (Gibco, U.S.A.) supplemented with 10% newborn calf serum, 100 u/mL penicillin and 100 u/mL streptomycin, and incubated at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air.

2.2 Preparation of irradiated samples

For the initial deproteinized DNA dsbs studies, the cells were removed from the culture dishes using 0.25% trypsinase, and suspended in PBS at a density of 1×10^7cells/mL in medium in a tube. An equal volume of prewarmed low melting point agarose in PBS (1%, type VII, pH8.0, Sigma) was added to the cell suspension. This final mixture was pipetted into plug moulds and kept at 4℃ for 1 h to allow the agarose set. Individual agarose plugs (Φ5 mm×2 mm) were incubated for 48 h at 50℃ in lysis buffer (1mg/mL proteinase K (Sigma), 1% SDS, 0.5 mol/L EDTA, 10 mmol/L Tris-HCl, pH 8.0) and kept in 0.5 mol/L EDTA (pH 8.0) at 4℃. Before irradiation the plugs were washed three times each for 6 h with TE (0.5 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 8.0) to remove the contaminating EDTA from the lysis buffer and transferred to ice cold PBS.

For cell survival, one day before irradiation, the cells were removed from the culture dishes using 0.25% trypsinase, and grown at a density of 5×10^4 cells/mL in medium in Φ35 mm petri dish.

2.3 Irradiation

A $^{12}C^{6+}$ ion beam (50 MeV/u) with 2.5 mm Spread Bragg Peak, supplied by the Heavy Ion Research Facility in Lanzhou (HIRFL) at IMP-CAS, was used to irradiate the samples at room temperature. The sample vessels were placed on a rotating sample holder with remote control, so as to irradiate one vessel per dose. Due to energy loss in vacuum window, air gap, organic glass (PMMA), and medium, the LET entering the samples was estimated at 125.5 keV/μm. For deproteinized DNA dsbs induction, the dose rate was adjusted to 4 Gy/min, for a dose range of 0–100 Gy. For the cell survival, the dose rate was adjusted to 3 Gy/min, for a dose range of 0–8 Gy.

2.4 Survival assay

After irradiation, the cells were plated at a density of about 100 surviving cells per 6-cm culture dish. Approximately 10 days later, cells were fixed and stained with Giemsa (pH7.0). Colonies of more than 50 cells were counted as survivors.

2.5 PFGE assay

The PFGE assay used in this study was applied as previously described[17]. The irradiated DNA plugs were inserted into wells of a 0.8% agarose gel (Sabc product). The gels were electrophoresed at 36 V for 72 h, the alternate time of positive and negative electric field was 5:1. Electrophoresis buffer was 0.5 × TBE (0.05 mol/L Tris, 0.05 M borate, 0.1 mmol/L EDTA; pH 8.4). Buffer temperature was maintained at 12°C by circulation through a cooling bath. DNA size

markers were included in each gel to calibrate DNA fragment size. These comprised yeast chromosomes from *Sacharomyces serevisiae* (size range of 225 Kbp to 2.2 Mbp). Following electrophoresis the gels were placed in 200 mL electrophoresis buffer with 0.5 μg/mL of ethidium bromide to stain DNA. The fluorescence pattern of DNA in ethidium-bromide stained gel was photographed and then the gel was performed densitometric scanning using fluorescent densitometer (CS-910 type, Shimadzu) with excitation wavelength of 254 nm and emission wavelength of 600 nm[18].

3 Results

3.1 Cell survival

The clonogenic survival curves in the four kinds of cell lines after ^{12}C ions irradiation were shown in Fig.1 in order to evaluate the cellular radio-sensitivity. The D_{50} (the dose necessary to reduce the survival to 50%) of B16, V79, HeLa and SMMC-7721 cells were 0.74, 1.70, 1.87 and 3.85, respectively. B16 was the most radiosensitive to ^{12}C ions, and V79 and HeLa had almost the same radiosensitivity.

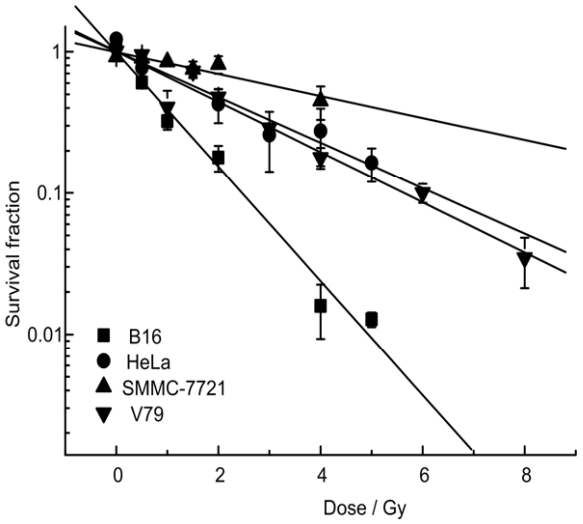

Fig.1 Survival curves of four kinds of mammalian cells irradiated with $^{12}C^{6+}$ ions (LET=125.5 keV/μm). The survival fraction was measured as the number of colonies formed after ^{12}C-irradiation. Error bars represent standard errors of the means. Date was fitted using a linear model.

3.2 Deproteinized DNA damage

The initial yield of the deproteinized DNA dsbs was expressed as the DNA double break level (DSBs/Mbp/Gy). Fig.2 showed the dose-response curves of four cell lines. The DNA double break level of all four cell lines increased linearly with radiation dose and values of slopes of the curves were ranged from 0.40 to 0.98. Concretely, the values for B16, SMMC-7721, HeLa and V79 were 0.40, 0.69, 0.82 and 0.98 (DSBs/100Mbp/Gy), respectively.

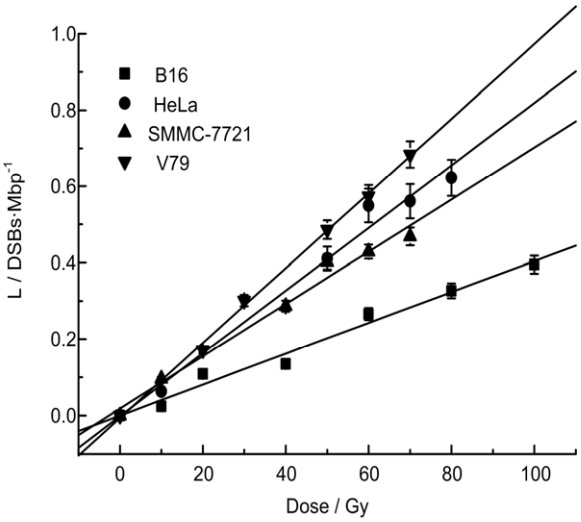

Fig.2 DSB in four kinds of deproteinizd DNA irradiated with with $^{12}C^{6+}$ ions (LET=125.5 keV/μm). DNA dsb was assessed using FIGE assay and expressed as the DNA break level (L). Error bars represent standard errors of the means.

3.3 Clonogenic radio-sensitivity vs deproteinized DNA dsbs

A comparison was made of the relationship between clonogenic radio-sensitivity and initial deproteinized DNA dsbs. The slopes of the initial DNA dsbs does-response curves for four cell lines showed a positive correlation with the survival parameter D_{50} (r=0.26658, fitting curve not shown) (Fig.3), it was inconsistent with the facts. From Figs.1 and 2, we found that there was an inverse relationship between dsbs and survival if the B16 cell line was not considered, so we analyzed the two parameters for three cell lines (SMMC-7721, HeLa and V79), and found that there was a correlation between them to a certain extent (r= –0.8717), but there was no statistical significance (p=0.326) (Fig.4).

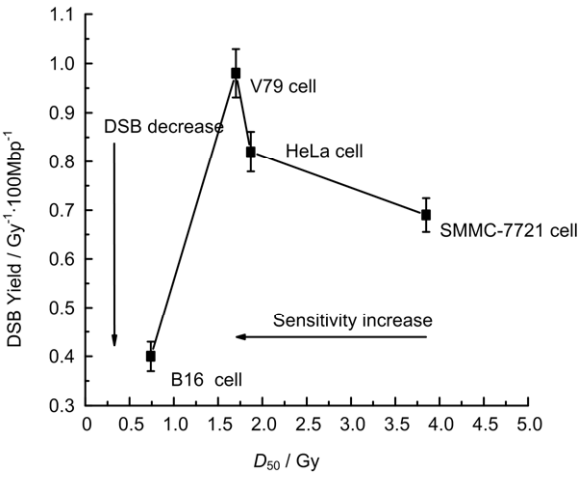

Fig.3 Relationship between clonogenic radio-sensitivity, measured as D50, and initial DNA dsbs slopes for four cell lines (B16, SMMC-7721, Hela and V79).

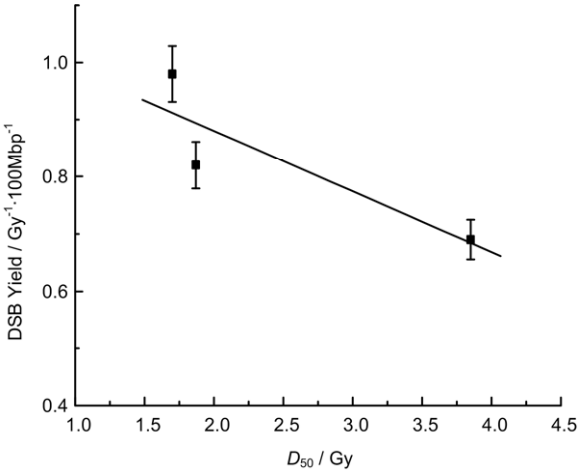

Fig.4 Relationship between clonogenic radio-sensitivity, measured as D50, and initial DNA dsbs slopes for three cell lines (SMMC-7721, HeLa and V79).

4 Discussion

The important radiobiological factor having a significant effect on tumor response to radiotherapy is intrinsic radio-sensitivity, which, always determined with a colony assay, is significantly associated with treatment outcome after radiotherapy. In this study, with this method to investigate the intrinsic radio-sensitivity of four cell lines, we found that the cell survival fractions decreased linearly with increasing dose (Fig.1). Judging from the value of the D_{50}, B16 was the most radio-sensitive to ^{12}C ions, V79 and HeLa had almost the same radio-sensitivity, and SMMC-7721 was the last (Fig.1).

It is generally accepted that DNA dsbs are the main type of damage that leads to the death of irradiated cells[19], and in view of the possible influence of chromatin structure on radio-sensitivity[11-16], we irradiated the deproteinized DNA of four kinds of cell lines with the ^{12}C ions, and analyzed the relationship between DNA dsbs and cell survival. A linear relationship was found between the dsb yield and dose. This confirmed the results in Refs.[20, 21]. Judging from the DNA dsb level, V79 cell was the most radio-sensitive to ^{12}C ions, HeLa cell was the second,and B16 was the last (Fig.2).

There are several reports in the literature indicating that radiation-induced DNA double strand breaks can be used as measures of intrinsic radio-sensitivity of tumour cells[5-8]. However, judging from the present results there was no correlation between the intrinsic radio-sensitivity of the four cell lines as determined by conventional methods and the radiation-induced DNA double strand breaks as determined by the PFGE (Fig.3), although there was a clear dose-response relationship for radiation-induced naked DNA double strand breaks. We noticed that there was an inverse relationship between dsbs and survival if the B16 cell line was not considered (Figs.1 and 2). Whether the relationship is related with the cell selection? We further analyzed the two parameters for three cell lines (SMMC-7721, HeLa and V79), and found that there was a correlation between them to a certain extent (r=–0.8717), but there was no statistical significance (p=0.326) (Fig.4). The reason for this discrepancy is not known, but the present results are in agreement with those in Refs.[5,6,9,22], where a complete lack of correlation was reported between the intrinsic radio-sensitivity, as judged by the clonogenic assay, and DNA double strand break induction in various human tumor cell lines.

It is well know that PFGE separates DNA fragments in an agarose gel using the negative charge according to their size. Most studies simply measured the amount of DNA that leaves the well and not the DNA that migrates along the lane itself[23, 24], which may account for the discrepancy in the relationship of DNA dsbs and survival. In addition, the resolution of the method used here may be not sufficient to measure accurately effects resulting in DNA fragments, because the upper separation limit of PFGE is around 15 megabase pairs (Mbp), and this approach is based

on the assumption of randomly distributed DSBs. Non-random distribution of dsbs on the nucleosomal level cannot be detected by PFGE due to limitations of the PFGE assay[24,25]. Or, not the initial dsbs, but the unrepaired dsbs is related to the clonogenic radiosensitivity[26]. Further studies would need to be done.

Taken together, the results clearly indicate that radiation-induced initial naked DNA double strand breaks are poor indicators of the intrinsic radiosensitivity of tumor cells irradiated *in vitro*.

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