Iran. J. Radiat. Res., 2006; 4 (3): 137-141

Track detection on the cells exposed to high LET heavy-ions by CR-39 plastic and terminal deoxynucleotidyl transferase (TdT)

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Background: The fatal effect of ionizing radiation on cells depends on Linear Energy Transfer (LET) level. The distribution of ionizing radiation is sparse and homogeneous for low LET radiations such as X or γ, but it is dense and concentrated for high LET radiation such as heavy-ions radiation. ***Materials and Methods:*** Chinese hamster ovary cells (CHO-K1) were exposed to 4 Gy Fe-ion 2000 keV/µm. The CR-39 is a special and sensitive plastic used to verify exact position of heavy-ions traversal. Terminal deoxynucleotidyl transferase (TdT) is an enzyme labeled with [^{3}H] dATP for detection of cellular DNA damage by autoradiography assay. ***Results:*** The track of heavy ions traversals presented by pit size was almost similar for all different doses of radiation. No pits to show the track of traversal were found in 20% of the cell nuclei of the irradiation. Apparently these fractions of cells wave not hit by heavy ions. ***Conclusion:*** This study indicated the possible usefulness of both the CR-39 plastics and DNA labeling with TdT method for evaluating the biological effect of heavy-ions in comparison with low LET ionizing radiation. **Iran. J. Radiat. Res., 2006; 4 (3): 137-141**

Keywords: *Track detector, heavy ions, CR-39 plastic, TdT.*

INTRODUCTION

The initial physical features of ionizing radiations are a major determinant of their

final biological consequences. Studies on the comparative effects of high and low-LET radiation on cultured cells could elucidate the mechanism of ionizing radiation effect on living cells, and to establish the radiobiological basis for the high LET radiotherapy of cancer [1]. Cosmic radiation is one of the main hazards for space exploration. High energy heavy-ions such as iron (Fe)-ions are important components of cosmic rays [2]. Uncertainty in radiation risk estimates for crews of long term mission are very high and direct biological measurements are necessary [3]. It is well know that the cell killing effect of ionizing radiation depends on LET and that RBE reaches a maximum at LET around 200 keV/µm [4], and then decreases abruptly, further declining to less than 1.0 with regard to very high LET heavy-ions such as ^{40}Ar or ^{56}Fe (LET>1000 keV/µm) [5, 6]. The ion clusters produced by high-LET radiation are not uniformly distributed. The incidence of non-hit cell events is higher in high-LET radiation than in the case of low-LET radiation. This fact could explain the decrease in the cell killing effect at higher levels of LET irradiation. Other situations rather than low-LET radiation has been pointed out with regard to very high-LET radiation by Goodhead *et al.* [7]; an individual cell which survives irradiation with very high-LET ions may either have received no lethal lesions despite having been intersected by one or more tracks or it may have been missed entirely by the tracks. Since the cell killing effect may be related to the nuclear traversal of heavy-ions, it is necessary to establish methods to distinguish the hit cells from the non-hit cells, especially with high-LET irradiation.

Ionizing radiation produces hundreds of different simple chemical products in DNA, and multitudes of possible clustered combinations. The simple products can

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include the single-strand and initial double-strand breaks. Track structure analysis has revealed that clustered DNA damage of severity greater than simple double-strand breaks is likely to occur at biologically relevant frequencies with all ionizing radiations [8]. In the high LET radiation, such as heavy-ions which characterized by a very localized microscopic distribution of hit, it is important to provide a useful tool to introduce hit site and DNA damage in restricted regions of cell nucleus [9].

In order to verify the possibility of cell killing effect related to the cell nucleus traversal of heavy-ions, we identified cellular sites (nuclear or cytoplasm) through which the heavy-ions traversed by CR-39 plastic and terminal deoxynucleotidyl transferase (TdT).

MATERIALS AND METHODS

Chinese hamster ovary cells (CHO-K1) (RIKEN cell bank, Japan) was used in this study. The cells were cultured in a flask with Ham's F12 medium supplemented with 10% fetal calf serum (Sigma) at 37°C in humidified 5% CO_2 atmosphere. Iron (Fe)-ions were accelerated by the RIKEN (The Institute of physical and chemical research, Japan) Ring cyclotron and the LET at the target cell site was adjusted to 2000 keV/µm. For the exposure of cells to heavy-ions, the beam-ions were injected from the bottom side of the cell in the culture flask (Nunc T25). The dose and LET distribution were measured with a parallel-plate ionizing chamber and a proportional counter filled with air and tissue-equivalent gas. The dose-averaged LET was controlled by inserting a rang shifter with the appropriate thickness. The details of the beam transport system and dosimetry have all been reported previously [10, 11]. As the first step for the analysis of the biological effects of the accelerated heavy-ions radiation we established a method for the position of ion hits within cultured CHO cells. The cell hit was physically determined using CR-39 plastic (solid state nuclear track detector: Harzlas/TNF-1) for detecting the exact position of heavy-ions traversals. When accelerated Fe-ions interacted with CR-39 plastic they could be registered as latent tracks [12, 13]. The CR-39-plastic was used in several researches such as alpha contamination monitoring and for dosimetry [14, 15]. To amplify latent tracks, the CR-39 plastic, attached to the bottom surface of a culture flask, was treated with PEW (KOH: 15%, Ethanol: 65%, Water 20%) as an etching solution, for 10 min at 37°C.

Etching solution tested in different time and concentrations and finally optimized it to recorded values. After optimizing, the experiment was repeated for three times.

To detect cellular DNA damage, terminal deoxynucleotidyl transferase (TdT) enzyme was purchased from "Takara Biomedical". TdT enzyme dose not need a template for its reaction and it requires an oligodeoxy-nucleotide of at least three bases as primer [16, 17]. One unit is the amount of the enzyme that incorporates 1 nmol of [^{3}H] dATP into primer DNA in 2 hour at 37°C and pH of 7.2. The reaction was affected by the added bases (dATP) and the divalent cation in the buffer and the terminal structure of DNA. Generally, a buffer containing Ca^{2+} is suitable for the addition of dATP[18]. Reaction mixture for one unit of TdT was prepared using: 100 mM of Sodium cacodylate, 1mM of $CaCl_2$, 0.2 mM of DTT (Dithiothreitol), 0.2 mM of dATP (2`-Deoxyadenosine 5`-Triphosphate) (Merck, Sigma); 1 µCi/µl of [^{3}H]-dATP (Amersham) dissolved in 50% Ethanol. The cultured cells were incubated 2 hours with the reaction mixture. Then, they were washed with Tirton X-100 and methanol, twice and once, respectively. Autoradiography was used extensively to localize any small macromolecule, such as DNA, RNA, or protein as well as in micro autoradiography. The processes of cells treatment continued by cutting off the flask containing cell surface and dipping in autoradiographic emulsion (Sakura NR-M_2) at 45°C in dark room. β-rays exposure from ^{3}H-dATP at 4°C was for 15-20 days done. The development of latent image and staining with Aceto-orcein were done.

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After optimizing, the experiment was repeated for three times. The detail of cells' preparation for autoradiography is explained elsewhere [19].

RESULTS

Figure 1 shows the average number of heavy-ions traversals per 100 µm × 100 µm (10^4 µm^2) on the CR-39 plastics were exposed to 1, 2 and 4 Gy of Fe-ions (2000 keV/µm). The track of heavy-ions traversals was presented by pit size (ϕ: ~2 µm) which was almost the same for all different doses examined. For detection the position of heavy-ion traversal on the cells, two photographs were taken using a phase-contrast microscope within the same area with one focused on the cells at the culture flask and the other focused on the pits detected on the surface of the CR-39 plastic which was placed on the surface of the bottom side of a culture flask when the cells were exposed to 4 Gy accelerated Fe-ions. An example of a superimposed photograph is shown in figure 2. The hit or non-hit cells were judged by heavy-ion tracks (yellow-orange circles) through their nucleus (dark area). No pits to show the track of traversal were found in 20% of the cell nuclei after irradiation. Apparently these fractions of cells were not hit by heavy-ions. Also, the damage can be visualized by TdT enzyme

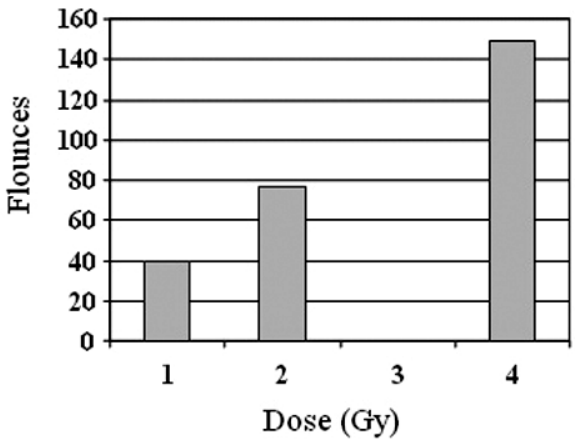


Figure 1. Relation between the fluences and dose in the CR-39 plastics exposed to Fe-ions.

that catalyzes the incorporation of deoxynucleotides into 3`-OH termini of single- or double- stranded DNA (figure 3). When cultured cells were incubated with TdT labeled [^{3}H] dATP which is a suitable isotopes for micro autoradiography, a low energy emitter ^{3}H in combination with a thin autoradiography emulsion gives high intracellular resolution. The β particles were released from Tritium entering the emulsion which coated the specimen in the dark and left it to expose. Figure 3 shows the specimen which are subsequently developed; a latent image in silver grains could be seen within the emulsion at the point where they stopped and released their energy in cells' nuclear or cytoplasm sites.

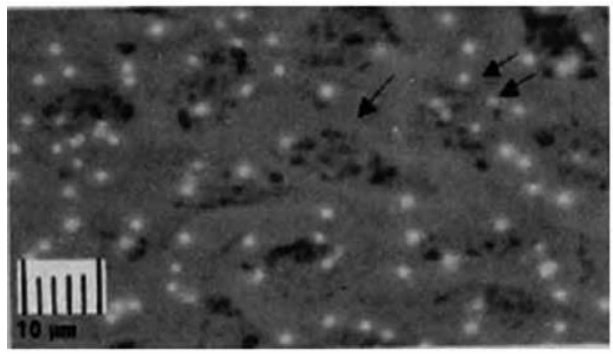


Figure 2. A superimposed photograph of CHO cells and CR-39 plastics exposed to 4 Gy Fe-ions. The single arrow shows a non-hit cell, double arrow show a hit cell.

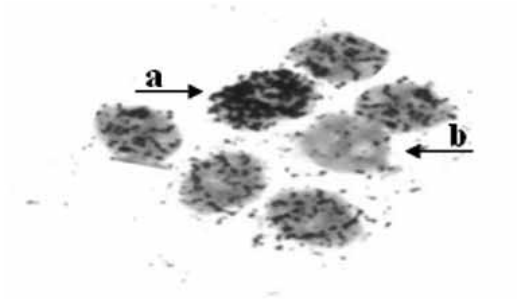


Figure 3. An example of [^{3}H]dATP incorporation into a CHO cells. (a) Typical densely labeled nuclei. (b) non-hit cell with negative labeled nuclei.

DISCUSSION

The potential application of heavy-ion therapy for clinical use, especially in cancer

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therapy, will be quite useful. This expectation is based on the biological characteristics of heavy charged particles and their precise dose-localization [20, 22]. It was shown that the cell survival could be measured as a function of the particle traversals through the cell nucleus or cytoplasm (figure 2). The biological response to heavy-ions differs from sparsely ionizing radiation, depending on the physical nature of the hit of heavy charged particles. Track structure induced heavy-ions by CR-39 plastics have been applied to understand aspects of radiation damage based upon fundamental physical and hit theory. No pits, presenting the track of traversal were observed in 20% of the cell nuclei after irradiation. This ratio has been in correlation with a previous study using time-lapse observation, where the occurrence possibility of division delay was studied (hit or non-hit) for individual cell during, before and after exposure to 4 Gy of Fe-ion [23]. In micro autoradiography assay, tritium was used at the cellular level. The released β particles had a mean range of about 1 μm in aqueous media, giving a very good resolution. Tritium-labeled compounds are usually less expensive than the ^{14}C or ^{35}S-labeled equivalents and have a longer half-life. The latent image was more stable at low temperatures and its sensitivity (signal versus background) might be improved by exposure in a refrigerator over desiccant. In some researches to investigate the mechanisms underlying the enhanced capacity for cell killing by high LET radiations, atomic force microscopy (AFM) is used to directly visualize and grade DNA fragments directly. These findings supported the hypothesis that the enhanced capacity for cell killing by high-LET radiation is due to clustering of multiple DNA double-strand breaks [24]. Recently, it became important to estimate the biological risks of high LET radiation encountered by space missions. The results of this research can be used for setting establishing health norms applied to the influence of radiation of different types, and for elaboration of measures to reduce health risks from space flight factors. High LET radiation enhanced apoptosis but not necrosis regardless of p53 status in human lung cancer cell lines [25]. Our previous results indicated that a PARP-IHC assay could be used for detection of heavy-ion hits to cell nuclei in p53 wild type (MGF) and also mutant type (CHO) cells. It seems that, during heavy-ion induction of DNA damage, there is no requirement for functional interaction between PARP and p53 proteins [26]. High-LET radiotherapy due to its higher biological effectiveness for cell killing and good localization is proposed; therefore, high-LET application might provide new insights in radiotherapy of patients carrying mutated p53 cancer cells. This paper presented two methods: 1) CR-39 plastics application to detect the cellular hit-sites (nuclear or cytoplasm) through which the heavy-ions traversed. We speculated that in heavy-ions exposed cells in the nucleus, the DNA molecule should be an important candidate for the target of cell lethality induced by heavy-ions. 2) Detection of DNA damage in exposed cells to heavy-ions by TdT enzyme assay scrutinizes application of theoretical approach of radiation damage of high LET in target molecules.

ACKNOWLEDGMENTS

The authors express their sincere gratitude to Dr. Y. Kobayashi and Dr. F. Yatagai for their valuable advices and thanks to RIKEN crew for providing us with the accelerated heavy-ions.

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