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Analysis of radiation-induced DNA double strand breaks after exposure to alpha particles: γ-H2AX staining method

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Abstract – The aim of this study was to analyse the γ-H2AX foci in HTB177 non-small lung cancer cells after irradiation with helium ions. Cells were irradiated in three different positions along the widened Bragg peak to follow formation of DNA DSB with respect to LET values. To compare diverse approaches in γ-H2AX foci analysis, we applied the method of foci quantification and total fluorescence intensity measurements. It was shown that helium ions significantly increased the number of γ-H2AX in all irradiated cells. Somewhat higher number of foci was found in samples irradiated within the LET of 24 keV/μm than in those exposed to 4.8 and 37 keV/μm. The same trend was observed after γ-H2AX total fluorescence analysis, showing a good correlation with the results of γ-H2AX foci counting. Further analysis of foci size, as well as colocalization with other DSB repair factors would complement these analyses and give more information about the nature of DNA lesions induced by helium ions.

INTRODUCTION

Irradiation provokes different types of DNA lesions, where double strand breaks (DSB) are considered as the most lethal. In response to DSB, repair factors are recruited to the lesion site and the process of the DNA repair is initiated [1]. One of the important proteins involved in DSB repair is the histone molecule H2AX. In presence of DSB, H2AX localises close to the lesion site where it becomes phosphorylated (γ-H2AX) and attracts other proteins involved in the repair. Known as an early DSB signalling molecule, γ-H2AX is frequently used as its marker B [1,2].

Fluorescent immunocytochemical labelling of proteins is a reliable tool for DNA damage analysis. It can be used to estimate the distribution of repair factors in injured cells [3]. This approach is commonly applied in γ-H2AX analysis, where foci counting is considered as the most precise. Another way for foci analysis is to measure intensity of the total fluorescence signal. This method is considered less precise comparing to foci counting, because of the background influence on the fluorescent signal [4].

Charged particle cancer therapy currently involves the application of protons and carbon ions [5]. These particles have favorable characteristics over conventionally used photon radiation: high linear energy transfer (LET) and a localized dose distribution. Moreover, they allow a precise dose-delivery to the tumour by reaching the dose maximum at the end of their path, in the Bragg peak region [5]. Among charged particles, helium ions are seen as a new approach for cancer treatment. They show good physical characteristics and could enable better sparing of surrounding tissue comparing to carbon ions. With respect to protons, helium ions show lower lateral scattering [6].

In this study we analysed formation of γ-H2AX foci in HTB177 cells after irradiation with helium ions of different LET. To compare diverse approaches in foci analysis, γ-H2AX quantification and total fluorescence measurements were made.

MATHERIALS AND METHODS

Cell culture

Non-small cell lung carcinoma cell line HTB177 was obtained from the American Type Culture Collection (ATCC, Manassas, Va, USA). Cells were grown in RPMI 1640 medium, supplemented with 10% foetal bovine serum (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 1% penicillin/streptomycin (Sigma-Aldrich Chemie GmbH), in humidified environment under optimal conditions with 5% CO_2 and 37°C (Heraeus, Hanau, Germany).

Irradiation procedure

Cells were seeded in slide flasks (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at appropriate cellular densities and maintained in standard conditions. On the next day, cells were irradiated with 62 MeV/u helium ions in three different positions along the somewhat widened Bragg peak. The irradiation positions were attained by interposing Perspex plates of different thickness before the cell monolayer, as previously described [7]. The values of LET for these irradiation positions were 4.8, 24 and 37 keV/μm. Samples were irradiated in pre-cooled growth medium on +4ºC [8]. The irradiation dose was 1 Gy with dose rate ~12 Gy/min. The procedure was carried out at 0° beam line at INFN-LNS in Catania, Italy.

Immunocytochemical γ-H2AX foci analysis

Cells were incubated for 0.5 h post-irradiation, in order to analyse the highest number of foci that could arise after irradiation [2]. After incubation, the growth medium was discarded, cells were rinsed with PBS and fixed in 4% PFA for 15 minutes at room temperature. The permeabilization solution 0.2% Triton-X in PBS was applied for 10 min at 4°C. Blocking and incubation with anti γ-H2AX antibody (Alexa Fluor 488, BioLegend Inc. San Diego, California, United States) was performed as described elsewhere [8]. Micrographs were obtained using laser confocal microscope Leica TCS SP5 II (Leica Microsystem CMS GmbH; Wetzlar, Germany) and processed with LAS AF Lite software (Leica Microsystem CMS GmbH). ImageJ Analysis PC software was used for foci quantification and measurements of total fluorescence intensity. Results are shown with respect to the control that was normalised to 1.

Statistical analysis

Statistical significance was determined with Student's t-test. Results are shown as means ± SEM, while the level of significance was $p < 0.05$.

RESULTS AND DISCUSSION

Quantitative γ-H2AX foci analysis

The HTB177 cells were irradiated with the dose of 1 Gy, as higher doses can cause signal overlap [9]. Irradiation with helium ions increased the number of γ-H2AX foci in all examined samples, around six times when compared to the control ($p<0.001$). Slightly higher number of foci was observed after irradiation with 24 keV/μm when compared to those at 4.8 and 37 keV/μm values of LET. However, no significant differences were found between these experimental groups (Figure 1).

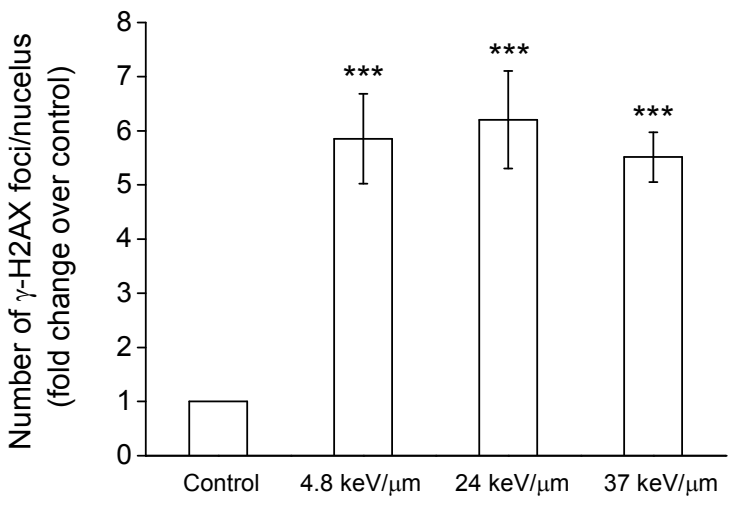

Figure 1. Quantitative analysis of γ-H2AX foci in HTB177 cells, performed 0.5 h after irradiation with helium ions of different LET (4.8, 24 and 37 keV/μm). Cells were irradiated with 1 Gy and results are presented as mean±SEM. *** $0.001 < p$.

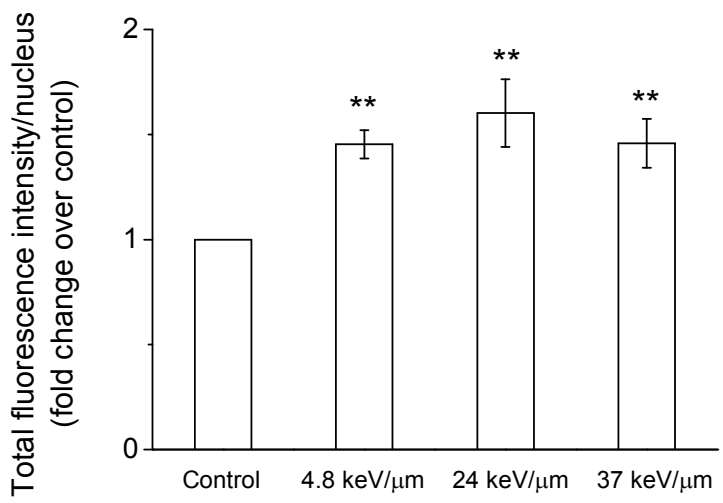

Figure 2. Analysis of γ-H2AX total fluorescence intensity measured per nucleus in HTB177 cells. Samples were irradiated with helium ions of different LET (4.8, 24 and 37 keV/μm). Analysis was performed 0.5 h after irradiation with 1 Gy and the results are presented as mean±SEM, ** $0.001 < p < 0.01$.

Total fluorescence analysis

Total fluorescence analysis showed a significant increase in fluorescent signal 0.5 h post-irradiation in all analysed samples ($p<0.01$). The intensity of fluorescence was the highest in samples irradiated within the 24 keV/μm value of LET, but no significant change was found between different irradiation positions (Figure 2). These findings are confirmed by γ-H2AX foci counting, meaning that there is a good correlation between these two approaches of data analysis.

CONCLUSIONS

Irradiation with helium ions significantly increased the number of γ-H2AX foci in HTB177 cells. Somewhat stronger response of HTB177 cells was noted after irradiation with LET of 24 keV/μm, comparing to 4.8 and 37 keV/μm values of LET. We found good correlation between quantitative γ-H2AX foci analysis and measurements of the total fluorescent signal. The further foci analyses regarding their size and colocalization with other repair proteins are needed for obtaining more information on their complexity.

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