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Investigation of the bystander effect in CHO-K1 cells

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ABSTRACT

Aim: Investigation of the bystander effect in Chinese Hamster Ovary cells (CHO-K1) co-cultured with cells irradiated in the dose range of 0.1–4 Gy of high LET ^{12}C ions and X-rays.
Background: The radiobiological effects of charged heavy particles on a cellular or molecular level are of fundamental importance in the field of biomedical applications, especially in hadron therapy and space radiation biology.
Materials and methods: A heavy ion ^{12}C beam from the Heavy Ion Laboratory of the University of Warsaw (HIL) was used to irradiate CHO-K1 cells. Cells were seeded in Petri dishes specially designed for irradiation purposes. Immediately after irradiation, cells were transferred into transwell culture insert dishes to enable co-culture of irradiated and non-irradiated cells. Cells from the membrane and well shared the medium but could not touch each other. To study bystander effects, a clonogenic survival assay was performed.
Results: The survival fraction of cells co-cultured with cells irradiated with ^{12}C ions and X-rays was not reduced.
Conclusions: The bystander effect was not observed in these studies.

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1. Background

Knowledge of the radiobiological effects of heavy ions at the cellular and molecular level is of fundamental importance in the field of radiation therapy. It has been accepted for a long time that the damaging effects of ionizing radiation are the results of the direct ionization of cell nuclei. However, in addition to effects in cells directly targeted with heavy ions, there is an evidence of non-targeted biological effects in cells that have not been directly irradiated. The bystander effect of heavy ions manifests itself as the loss of clonogenic potential, alterations in gene expression profiles, and the elevated frequency of micronuclei, which arise in non-irradiated cells having received signals from irradiated cells.[1] The phenomenon of radiation-induced bystander response was first described by Nagasawa and Little in 1992,[2] when increased frequencies of sister chromatid exchanges (SCEs) were observed in about 30% of the cells exposed to α-particle by which <1% of the nuclei was traversed by a single α-particle track.

The effect induced by irradiated cells and their progeny on neighboring non-irradiated cells was studied. CHO-K1 cells were irradiated with ^{12}C ions and X-rays with three different doses: 0.1 Gy, 1 Gy, 4 Gy. Investigation of the bystander effect was enabled by co-culture of irradiated and non-irradiated cells in special transwell dishes. Clonogenic survival assay was used in these studies.

2. Materials and methods

2.1. Cell line and culture conditions

Chinese Hamster Ovary cells (CHO-K1) were exposed to two kinds of ionizing radiation – high LET ^{12}C ions and X-rays. The cell line is characterized by genetic stability, the ability to form colonies and a relatively rapid growth rate, with a cell cycle of 12–14 h. The cells were cultured in 5A McCoy (Gibco, USA) medium, containing 10% fetal bovine serum (FBS) (Gibco, USA), 1% penicillin and streptomycin (Gibco, USA) and incubated in a humidified atmosphere at 37 °C with 5% CO_2.

2.2. Irradiation facility

The experimental set-up has been described previously[3] and therefore only a short review is presented. An experimental set-up with a horizontal heavy ion beam designed for radiobiological research at the Heavy Ion Laboratory of the University of Warsaw (HIL) was used. It provides the possibility to irradiate biological samples at room temperature and atmospheric pressure by various ions at high LET. A view of the facility is shown in Fig. 1.

At the entrance the beam is collimated by a 2 mm aperture. To achieve a homogeneous radiation field over the area of $1 \times 1\,cm^2$ of the exit window (made from havar with thickness of $2.3\,mg/cm^2$), the ion beam was passively spread out by a scattering gold foil (Goodfellow Cambridge Limited, Huntington, UK) with thickness of $13\,mg/cm^2$. The on-line ion beam monitoring is ensured by a silicon detector placed at 20°.

2.3. Irradiation

One day before irradiation cells were seeded on the Petri dish made with mylar bottom with thickness of 6 μm. To obtain homogeneous irradiation of all CHO-K1 cells, biological samples were fastened to a movable, specially designed sample holder mounted on an x–y–z stepping motor with remote control, set at a distance of 1.2 cm from the exit window. At this system the time dependence of the beam intensity during the sample irradiation as well as the energy spectrum of the scattered beam were registered. The signals from the monitor detector were counted in a fast programmable scaler.

When the number of registered particles reached the preset value (defining the dose), a start signal was created and the target changed its position according to a planned route. The data were visualized on-line at the PC monitor by a graphical interface. Communication between the PC and the electronics was via a CAMAC crate controller.

Primary energy of the carbon ions was 48 MeV. Scattering gold foil and havar exit window degraded the ions energy to 27 MeV. Air layer and mylar bottom of the Petri dish again reduced energy of the ions and in the result carbon ions with 17 MeV energy hit the cells. Linear energy transfer (LET), namely the average amount of energy deposited

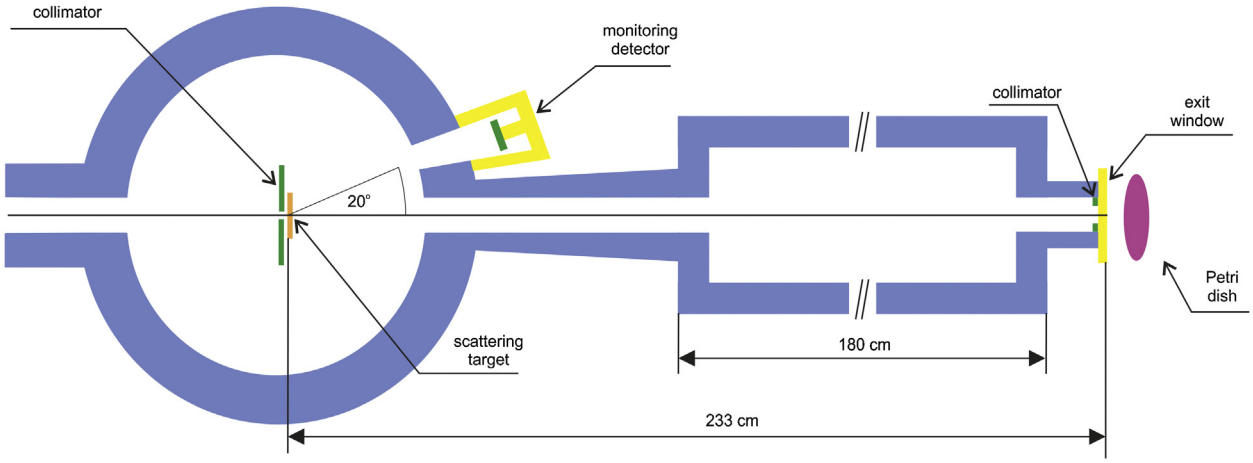

Fig. 1 – Schematic view of the set-up for radiobiological studies with the horizontal beam line.

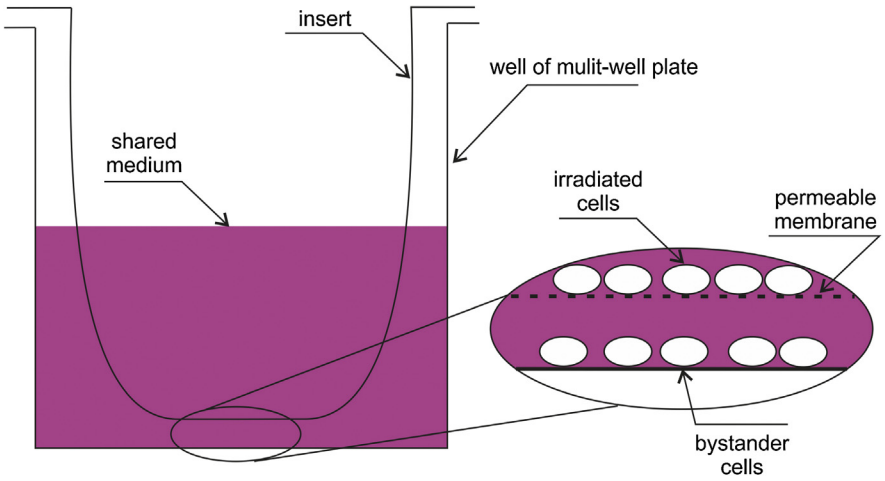

Fig. 2 – Scheme of transwell culture insert dish with permeable membrane.

per unit length was 640 keV/μm in the cell area. Time of the irradiation was comparable for all doses (300–400 s) and thus the dose rate was variable (from 0.3 Gy/min for dose of 0.1–0.8 Gy/min for 4 Gy dose). The ion fluence was also variable (from 9.8×10^4 ions/cm^2 for 0.1 Gy to 390×10^4 ions/cm^2 for 4 Gy).

Cells were irradiated with X-rays in the Institute of Nuclear Chemistry and Technology in Warsaw. Special Petri dishes with mylar as the bottom were used during irradiation and the dose rate was 1.14 Gy/min (Xylon International Smart 200-E irradiator, Xylon, San Jose, USA).

Immediately after irradiation, the cells were transferred into transwell culture insert dishes to enable a co-culture of irradiated and non-irradiated cells (Fig. 2). Since the diameter of the pores in the membrane inserts was 1 μm, cells from the membrane and well shared the medium but could not touch each other.

2.4. *Determination of bystander cell survival*

The clonogenic survival assay determines the ability of a cell to proliferate, thereby retaining its reproductive ability to form a colony. Bystander cells were plated at 150 cells/well, while irradiated cells were plated on the membrane of the insert with different densities: 10^3, 5×10^3 and 25×10^3 cells/insert. After 7 days of incubation, the medium was removed, the cells were washed with PBS and fixed with methanol for 10 min. Cells were stained with 20% Giemsa solution for 15 min and scanned in order to obtain the digital image. This allowed for further analysis without damaging the collected samples and to work with a much enlarged image of the colonies. Then, the number of colonies was counted with a specially created application. The results are expressed by the plating efficiency (PE) and survival fraction (SF). PE is calculated in control culture of cells and it is defined by the number of counted colonies/number of plated cells while SF is referred to irradiated and bystander cells and is defined by the number of counted colonies/(number of plated cells × PE).[4]

3. Results

Representative images of the stained CHO-K1 colonies are shown in Fig. 3. Plating efficiency of control CHO-K1 cells ranged between 80% and 97%, depending on the experiment.

Results for survival fraction for each sample are shown in Fig. 4. The error bars include errors related with plating cells (2%) and standard errors in accordance with the principle of the propagation of error. The clonogenic survival after irradiation with ions is determined as the average of four separate experiments in six repetitions per dose per density of cells in the inserts. The survival fraction of CHO-K1 cells co-cultured with cells irradiated with ions is close to 1 within errors, regardless of the absorbed dose and density of irradiated cells plated on inserts. The clonogenic survival after X-rays irradiation is the average of three separate experiments in six repetitions per dose per density of cells in the inserts. In the case of the cells co-cultured with cells irradiated with X-rays the survival fraction is slightly lower than 1 for higher doses.

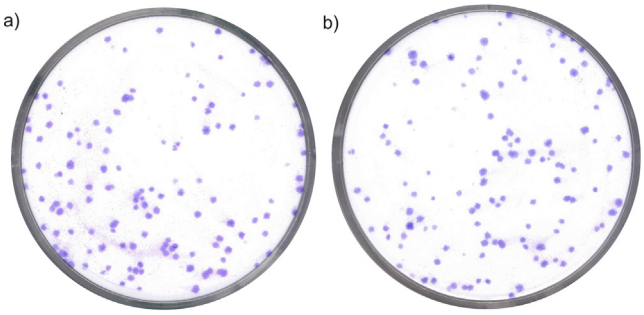

Fig. 3 – Colonies of control cells co-cultured with 1000 non-irradiated cells (a) and colonies of bystander cells co-cultured with 1000 cells irradiated with X-rays (b).

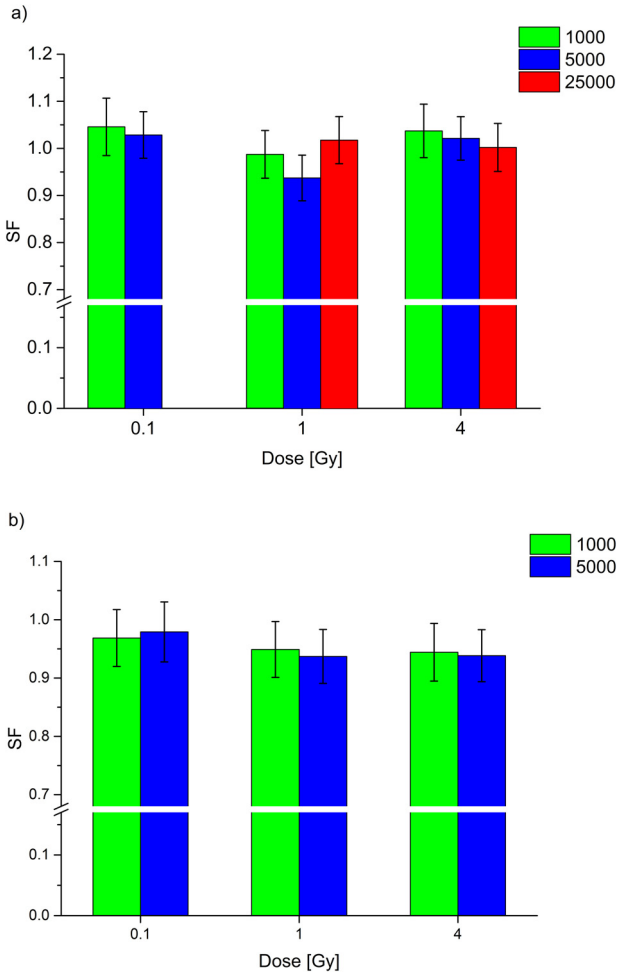

Fig. 4 – Survival fraction of cells co-cultured with cells irradiated with ^{12}C ions (a) and X-rays (b), plated at different densities in inserts.

4. Discussion and conclusion

The radiation induced bystander effect is a non-targeted effect where a signal produced by a directly irradiated cell can induce a subsequent response in non-irradiated cells. The responses that have been measured include micronucleus formation, increased sister chromatid exchanges (SCE), chromosomal rearrangements, gene mutations, genomic instability, epigenetic changes such as DNA methylation, histone modification and RNA-associated silencing, cell killing, decreased cell survival and a variety of damage-inducible stress responses (see for review 5–8). Bystander effects have been observed with both high- and low-linear energy transfer (LET) radiations.[9–11] Although the existence of bystander effects is incontrovertible, the physical and biological parameters that characterize bystander responses as well as underlying mechanisms are yet to be clearly defined. Thus, our study was designed to determine cell survival of bystander cells co-cultured with cells directly exposed to 0.1 Gy, 1 Gy and 4 Gy of ^{12}C ions and X-rays. After irradiation, cells were immediately transferred into transwell culture insert dishes to enable co-culture of irradiated and non-irradiated cells. Non-irradiated cells were plated at 150 cells/well, while irradiated cells were plated with three different densities: 10^3, 5×10^3 and 25×10^3 cells/insert. Summarizing our collected data, the survival fraction of CHO-K1 cells co-cultured with cells irradiated with different doses of ^{12}C ions and X-rays was not reduced regardless of the absorbed dose and density of irradiated cells plated on inserts. Thus we found no evidence for bystander effect in our experiments. Our results are in conflict with a number of published results.[7,8] However, there are also data in the literature showing no evidence of a bystander effect in a variety of cell lines, including clonogenic survival, induction of chromatid breaks and micronuclei.[12–14] It is unclear why a bystander effect was not observed in the experiments reported here. One possible explanation for these results may be the fact that the CHO-K1 cells do not produce a bystander signal or they do not respond to the bystander signal produced under experimental conditions applied in our study. Dependence of bystander effect on cell type and experimental conditions was reported in the literature.[15–17] Moreover, our previous studies of bystander cell survival using the medium transfer technique after exposure of CHO-K1 cells to ^{60}Co irradiation revealed the occurrence of the bystander effect in non-irradiated CHO-K1 cells plated with density of 300 cells per dish, but did not confirm the reduction of the survival fraction of bystander cells plated with density of 700 cells per dish.[18] It was demonstrated that cell density influences experimental conditions by depriving cells of serum, glucose or oxygen which have a variable influence on their growth and survival.[19] Depriving cells of serum as well as specific serum batch may inhibit/elicit production of, or response to the bystander signal, as was presented by Mendoca et al.[20] who found that the use of growth medium supplemented with a specific lot of calf serum was capable of increasing the number of cells undergoing radiation-induced transformation. An alternative explanations for the observed lack of evidence for radiation-induced bystander response in our study may include dependence on radiation quality, dose, and/or LET, interaction effects between bystander effects and radioadaptive responses.[8,17] Experiments designed specifically to test these hypotheses would be necessary to evaluate such suppositions.

Conflict of interest

None declared.

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REFERENCES

1. Hamada N. Recent insights into the biological action of heavy-ion radiation. *J Radiat Res* 2009;**50**:1–9.
2. Nagasawa H, Little JB. Induction of sister chromatid exchanges by extremely low doses of α-particles. *Cancer Res* 1992;**52**:6394–6.
3. Czub J, Banaś D, Braziewicz J, et al. An irradiation facility with a horizontal beam for radiobiological studies. *Radiat Prot Dosim* 2006;**122**:207–9.
4. Hall EJ. *Radiobiology for the radiologist*. Philadelphia, New York: Lippincott Williams & Wilkins Publishers; 2000.
5. Morgan WF. Non-targeted and delayed effects of exposure to ionizing radiation: I. Radiation-induced genomic instability and bystander effects in vitro. Review. *Rad Res* 2003;**159**:567–80.
6. Morgan WF. Non-targeted and delayed effects of exposure to ionizing radiation: II. Radiation-induced genomic instability and bystander effects in vitro, clastogenic factors and transgenerational effects. Review. *Rad Res* 2003;**159**:581–96.
7. Mothersill C, Seymour C. Radiation-induced bystander effects and the DNA paradigm: an "out of field" perspective. Review. *Mutat Res* 2006;**597**:5–10.
8. Blyth BJ, Sykes PJ. Radiation-induced bystander effects: What are they and how relevant are they to human radiation exposures? Review. *Rad Res* 2011;**176**:139–57.
9. Iyer R, Lehnert BE. Factors underlying the cell growth-related bystander responses to α particles. *Cancer Res* 2000;**60**:1290–8.
10. Yang H, Asaad N, Held K. Medium-mediated intercellular communication is involved in bystander responses of X-irradiated normal human fibroblasts. *Oncogene* 2005;**24**:2096–103.
11. Yang H, Magpayo N, Rusek A, Chiang I-H, Sivertz M, Held KD. Effects of very low fluencies of high-energy protons or iron ions on irradiated and bystander cells. *Rad Res* 2011;**176**:695–705.
12. Groesser T, Cooper B, Rydberg B. Lack of bystander effects from high-LET radiation for early cytogenetic end points. *Rad Res* 2008;**170**:794–802.
13. Zyuzikov NA, Coates PJ, Parry JM, Lorimore SA, Wright EG. Lack of non-targeted effects in murine bone marrow after low-dose in vivo X irradiation. *Rad Res* 2011;**175**(3):322–7.
14. Fournier C, Barberet P, Pouthier T, et al. No evidence for DNA and early cytogenetic damage in bystander cells after heavy-ion microirradiation at two facilities. *Rad Res* 2009;**171**:530–40.
15. Mothersill C, Seymour C. Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *Int J Rad Biol* 1997;**71**:421–7.
16. Mothersill C, Rea D, Wright EG, et al. Individual variation in the production of a bystander signal following irradiation of primary cultures of normal human urothelium. *Carcinogenesis* 2001;**22**:1465–71.
17. Sowa MB, Goetz W, Baulch JE, et al. Lack of evidence for low-LET radiation induced bystander response in normal human fibroblasts and colon carcinoma cells. *Int J Radiat Biol* 2010;**86**:102–13.
18. Czub J, Banaś D, Braziewicz J, et al. *Biologiczne oddziaływanie promieniowania. Raport roczny Instytutu Fizyki Uniwersytetu Jana Kochanowskiego w Kielcach*; 2010. p. 23 (in Polish only) http://www.ujk.edu.pl/ifiz/pl/files/reports/RAP10.pdf
19. Johnson WEB. The influence of serum, glucose and oxygen on intervertebral disc cell growth in vitro: implications for degenerative disc disease. *Arthritis Res Ther* 2008;**10**:R46.
20. Mendonca MS, Temples TM, Farrington DL, Bloch C. Evidence for a role of delayed death and genomic instability in radiation-induced neoplastic transformation of human hybrid cells. *Int J Radiat Res* 1998;**74**:755–64.