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Original Research Article

Evaluation of low-dose proton beam radiation efficiency in MIA PaCa-2 pancreatic cancer cell line vitality and H2AX formation

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ABSTRACT

Background and objective: The aim of this study was to evaluate the efficiency of proton beam irradiation in pancreatic cancer cell line MIA PaCa-2 and its role in the cell cycle, apoptosis, and formation of histone γH2AX in different reparation times (72-h follow-up).
Material and methods: The MIA PaCa-2 pancreatic carcinoma cell line was irradiated with 1.6-Gy proton beam. After irradiation, cell viability was measured colorimetrically, and the cell cycle, apoptosis, and γH2AX expression were evaluated on a FACScan cytometer.
Results: Low-dose proton beam irradiation had an effect on the MIA PaCa-2 tumor cell line already 1 h after exposure, but maximal lethality was reached after 72 h postirradiation with a cell viability rate of 24%. The cell cycle went into partial G1/0 arrest, and was released after 72 h. The expression of γH2AX was strong and its levels were significantly elevated as late as 48 h post radiation. The apoptosis levels increased with post radiation incubation time to reach 79% after 72 h.
Conclusions: Our data demonstrate that low-doses proton beam irradiation had an effect on MIA PaCa-2 pancreatic carcinoma cell line. Full extent of irradiation had an impact only 24 h postirradiation, triggering DNA arrested cell cycle in G1/0 phase. Formed DNA DSBs were found to be repaired via the NHEJ pathway mechanism within 72 h. Unsuccessful repaired

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DSBs induced apoptotic cell death. After 72 h reparation processes were completed, and cell cycle was released from arrest in G1/0 phase.

1. Introduction

Pancreatic carcinoma possesses one of the highest lethality and incidence rates and is highly drug-resistant. While the incidence rates have been declining for many types of cancer, the incidence rate for pancreatic cancer is increasing and it is one of the few cancers for which the relative survival rate has not improved substantially during 40 years [1]. Treatment options for pancreatic cancer are limited. Only 15% of patients are eligible for surgical tumor removal. Chemotherapy or chemoradiotherapy may be offered before or after surgery, or as the only treatment.

Proton therapy is an effective form of radiation therapy with fewer side effects [2,3]. The use of proton beam in radiation therapy has increased considerably in the past few years, but the first data about proton beam use in medical treatment was published in 1946 by Robert R. Wilson, who is considered ''a father of proton therapy'' [4].

Proton beam radiation favorable usage in cancer therapy is based on its physical features. This ionizing radiation allows for dose escalation (because of very limited radiation outside of the target zone) which improves local tumor control in anatomic sites where local control is suboptimal with standard treatments. Improved dosage concentration reduces damage to healthy tissue, resulting in lower acute and late toxicity [3,5].

Proton beam therapy has been applied to patients with tumors in clinical studies since 1961 [6], but it is still unknown which cancer patients would benefit from proton beams more than from standard radiotherapy. Details of proton radiation effect on pancreatic carcinoma are unknown and information on chemotherapy usage with proton beam radiation is very limited [7,8].

Cellular exposure to ionizing radiation leads to oxidizing events that damage DNA structure [9,10]. The increased reactive oxygen species S (RO) level in cell induces DNA damage in the form of double strand breaks (DSBs), which in turn stimulate cells to activate a number of various DNA repair mechanisms. Unrepaired and misrepaired DSBs are serious threats to the genomic integrity [11,12] such as chromosomal aberrations which can simultaneously affect many genes and cause cell death.

One of the sensitive assays in radiobiological studies on DNA damage response is immunofluorescent staining with anti-γH2AX antibody. This technique reveals an early step in cell response to DSBs: rapid phosphorylation of the histone H2AX at Ser139, resulting in γ-H2AX accumulation at the damage sites [13].

The aim of this study was to investigate the effect of proton beams on pancreatic carcinoma in an attempt to gain insight into the mechanisms of proton radiation-induced cell death. In the present paper, the authors report the effect of low-dose proton beam irradiation (1.6 Gy) on cell cycle, apoptosis, and kinetics of DNA damage at various time points after irradiation in MIA PaCa-2 pancreatic cancer cell line.

2. Materials and methods

2.1. Cell culture

The MIA PaCa-2 pancreatic carcinoma cell line was obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany) and 1% antibiotics: 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen) at 37 °C in humidified atmosphere (5% CO_2). Cells were subcultured following the standard protocol.

2.2. Experimental design

Twenty-four hours before to irradiation, cells were seeded on 24-well (200,000 cells for γH2AX assay and 50,000 for apoptosis and cell cycle assays) and 96-well plates (10,000 cells for cell viability assay). Radiation treatments were performed on 80% confluent cell cultures. After treatment, cells were returned to the incubator for additional 1, 3, 6, 24, 48 or 72 h. The control group was untreated with proton irradiation.

All radiation procedures were accomplished in the Laboratory of Nuclear and Environmental Radioactivity Research, Institute of Physics. Cells were exposed in vitro to 20 nA and 1.6-MeV proton irradiation at a dose of 1.6 Gy [14]. Biological effects were evaluated in the National Cancer Institute.

2.3. Cell viability assay

To assess cell viability, 1% crystal violet solution stain was used. Cells were incubated and treated in 96-well flat-bottomed plates. At designed time points DMEM medium was removed, cells were washed and fixed with subsequent usage of 70% and 96% C_2H_5OH for 10 min each. Fixed cells were stained with 1% crystal violet dye for 15–20 min in room temperature. Stain was removed and cells were incubated with 0.2% Triton-X solution overnight in 37 °C. Next day solution was moved to a clean plate. Optical density was measured at 630 nm using micro-plate reader ELX 808 IU (Biotek). Each condition was tested 3 times, and results were expressed as mean ± standard deviation. Optical density of control samples was treated as 100% for further calculations.

2.4. FITC-annexin V/PI staining and cell cycle analysis

Apoptosis-mediated cell death and cell cycles of tumor cells were examined using a double staining method with

FITC-labeled Annexin-V (Invitrogen) and Propidium Iodide (PI, Sigma–Aldrich) as previously described [15]. Fluorescent cells were analyzed using a FACSort flow cytometer with a 488-nm excitation laser (Becton Dickinson, San Jose, CA, USA). Each experiment was repeated 3 times.

2.5. *Immunofluorescent detection of γ-H2AX*

Double strand breaks were detected by using monoclonal mouse, anti-human γH2AX (Abcam) as primary antibody. After treatment control and treated cells were washed with PBS and fixed using CellFIX (Becton Dickinson, San Jose, CA, USA) for 10 min at 4 °C. Afterwards cells were centrifuged (10 min, 400 × *g*), washed and permeabilized using 0.2% Triton-X/PBS for 4 min on ice. Cells were then incubated with 250 μL (1:500 dilution) of anti-γH2AX antibody for 30 min, washed and incubated with 250 μL anti-IgG secondary antibody (1:400 dilution) for 30 min (goat, anti-mouse, Abcam) at room temperature in the dark [14,16]. The level of fluorescence in control and irradiated cells were estimated by counting a minimum of 10,000 cells using FACSort flow cytometer (Becton Dickinson, San Jose, CA, USA).

3. Results

3.1. *Viability*

After proton irradiation, viability of MIA PaCa2 cells was measured in several time points: after 1, 3, 6, 24, 48 and 72 h. Viability of the cells before the irradiation procedure was used as reference (100%). The results in Fig. 1 show that viability of the cells decreased in time after the irradiation procedure. Viability of the cells was 87% ± 1% at 1 h and 6 h, and 42%, 36%, and 24% at the time points of 24 h, 48 h and 72 h, respectively. Samples taken at 1 h, 3 h and 6 h after irradiation were not statistically different from either the control ($P > 0.05$) or each other ($P > 0.05$). Each measurement at 24–72 h after irradiation

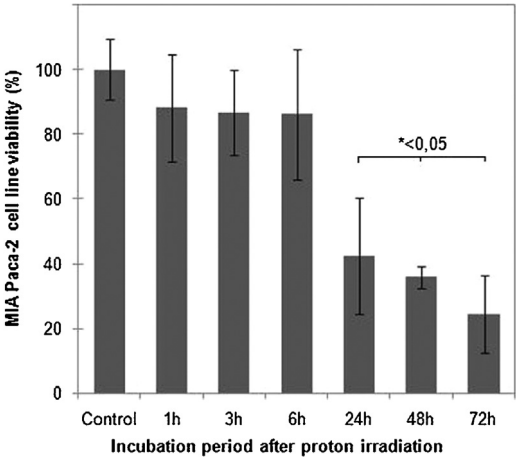

Fig. 1 – Viability of MIA PaCa2 cell line after proton irradiation. Measurements were performed at different time points. Error bars indicate standard deviation. *Student *t* test, statistically significant when compared with the control value.

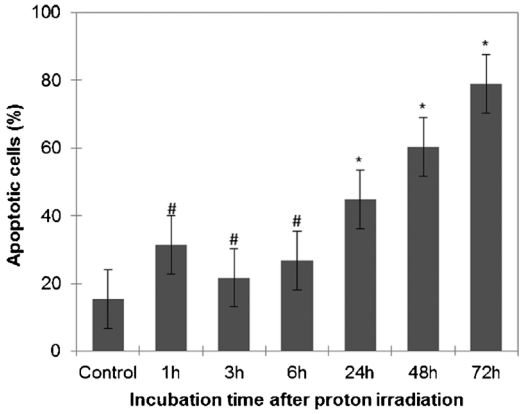

Fig. 2 – Apoptotic cells in MIA PaCa-2 cell line after proton irradiation measured at specified time points. Error bars indicate standard deviation. #P > 0.05 (not significant), *P < 0.01 as compared to the control value, Student *t* test.

was statistically significant from the control ($P < 0.001$); however, they were all statistically not significant comparing each other ($P > 0.05$).

3.2. *Apoptosis and cell cycle*

Fig. 2 displays the percentage of apoptotic MIA PaCa-2 cells at various time points after proton irradiation. There were no significant differences in the percentage of apoptotic cells at 1 h, 3 h and 6 h after irradiation when compared with the control ($P > 0.05$). Moreover, the percentages at 1 h and 6 h were not different from each other ($P = 0.23$). The percentage of apoptotic cells 24 h after irradiation was 45%; after 48 h, 60%, and after 72 h, 79%; the differences were statistically significant when compared with the control ($P < 0.01$) and between each other ($P < 0.02$).

Fig. 3 depicts changes in the cell cycle of MIA PaCa-2 cells at different time points after irradiation. Temporary G1/0 cell cycle arrest was observed in the irradiated sample after 24 h and 48 h, which was released after 72 h. In the control sample, a drastic increase in G1/0 phase of the cycle was noted after 72 h.

3.3. *γH2AX expression and kinetic measurements*

The expression of γH2AX induced by proton irradiation was evaluated at subsequent time points (Fig. 4). The initial response was very high with 97% of the cells expressing the protein at 1 h after radiation and gradually decreasing to 93.43%, 83.47%, 62.3%, 23.1%, and 3.78% at 3 h, 6 h, 24 h, 48 h, and 72 h, respectively. Each measurement was statistically different from the control ($P < 0.01$) and between each other ($P < 0.02$) with the exception of the last measurement at 72 h after radiation, which was not significantly different from the control ($P = 0.963$).

4. Discussion

It is well known that even low-dose irradiation has numerous biological effects on cells, including cell survival, apoptosis

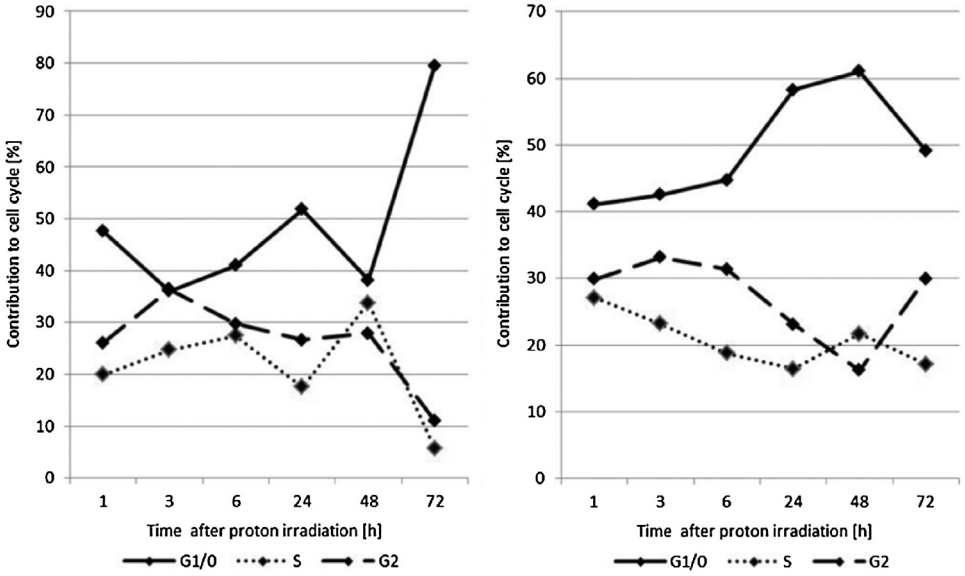

Fig. 3 – Cell cycle changes in control (left) and irradiated (right) cells.

induction, delayed cell death, etc. Low-dose irradiation is important for patients receiving cancer therapy due to reduction of risk of complications and side effects. In our experiment we investigate low-dose proton beam irradiation in the MIA PaCa2 cancer cell line. Data showed that the effects of irradiation on MIA PaCa2 cell line viability were noticeable already at 1 h after irradiation, but only at 24 h after the procedure, cell survival differed statistically significantly from the control and decreased sharply at following time points. The tendency of decreasing viability from 24 h to 72 h postirradiation was statistically insignificant; however, it was strongly supported by a significantly increasing proportion of apoptotic cells at this time point.

Ionizing radiation induces DNA damage triggering a p53-mediated response [17]. If double-strand breaks (DSBs) are abundant, p53 response blocks the cells ability to divide and proliferate [18]. If left unrepaired, DSBs can result in permanent cell cycle arrest, induction of apoptosis, or mitotic cell death caused by loss of genomic material. DSBs repair proceeds through two genetically different pathways: homologous recombination (HR) and non-homologous end joining (NHEJ) [19]. These DNA repair mechanisms can be differentiated through cell cycle analysis.

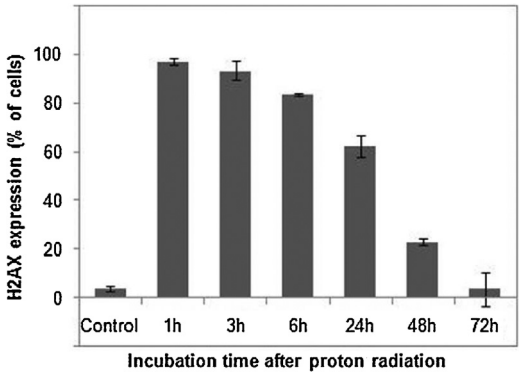

Fig. 4 – Phosphorylation of histone H2AX in MIA PaCa-2 cells after proton irradiation measured at different time points. Error bars indicate standard deviation.

The cell cycle can be stopped in G1/0 or S phase, which indicates the repair via the NHEJ pathway, or it can be stopped in G2 phase, which points to the HR pathway [17,20]. Regardless of the approach, if the repair is unsuccessful, it leads to apoptotic cell death [12,20]. Our results revealed an early step in cell response to DSBs and their reparation kinetics after low-dose proton beam irradiation.

The results show that low-dose proton beam irradiation in the MIA PaCa2 cell line triggered DSBs in the DNA, resulting in cell cycle arrest after 24 h in G1/0 phase. This is characteristic of the NHEJ reparation pathway. The cell cycle arrest in G1/0 phase gives time for cells to repair damage to DNA before replication occurs to avoid genetic lesions in progeny cells. Based on our results, it is worth noting that cell cycle arrest was not complete and was released after 72 h, once DSBs were repaired, and the cells with unrepairable damage underwent apoptosis.

Our data show that G1/0 cell cycle arrest occurred in the control after 72 h, i.e., when the culture reached confluence and there was no room for the cells to multiply. The irradiated culture did not experience cell cycle arrest after 72 h because of dramatically decreased viability and slower growth caused by cell cycle arrest prevented the culture from filling the culture dish. Our data on γH2AX showed clearly elevated levels, and this is a certain sign of DSBs as well as of reparation efforts undertaken by the cell [13]. Strongest presence of γH2AX already at 1 h after irradiation showed that DSB were formed immediately after the irradiation procedure. Those levels decreased in time and reached the control level after 72 h, when, as it can be assumed, all DSB repair procedures were finished. Confirmation of the finished repair process was provided by the released cell cycle after 72 h.

Further work would be appropriate to determine the direct molecular and biochemical processes and exploit them for a potential therapeutic effect.

5. Conclusions

Low-dose proton beam radiation had an obvious effect on the MIA PaCa-2 cell line, a laboratory model for pancreatic carcinoma. Viability data are in line with the data on apoptosis and showed gradually increasing cell death in the irradiated cultures up to 72 h after exposure. Recruitment of repair proteins began immediately after radiation as demonstrated by γH2AX mobilization; however, cell cycle arrest and, therefore, the majority of repair processes started only at 24 h after irradiation. Low-dose proton beam irradiation triggered DNA DSBs and arrested cell cycle. DSBs were repaired via the NHEJ pathway within 72 h. After 72 h, DSB repair processes stopped with released cell cycle and γH2AX levels comparable to the control.

Acknowledgments

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