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Radio-adaptive doses effect on HT29 and MRC5 cell lines: comparison in hypo and hyper fractionation regime

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► ***Original article***

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ABSTRACT

Background: The exposure of cell lines to low-dose irradiation leads to changes at molecular level, which may induce adaptive response. We examined radio-adaptive doses effect on human colorectal adenocarcinoma cell line (HT29) and human fetal fibroblasts (MRC5) cell line followed by hyper and hypo fractionation regimes, with main purpose to decrease cell viability in HT29, and at the same time to spare MRC5 cells. ***Material and Methods:*** The cell lines were pre-irradiated with 0.03Gy, 0.05Gy and 0.07Gy. Two hours later, control and pre-irradiated cells were irradiated in hyper and hypo fractionation regimes. Cell viability and the total cell number were measured. ***Results:*** Comparing the response between two cell lines in the same regime, it was found that pre-irradiation dose of 0.05Gy increased cell viability in MRC5 cell line, accompanied with decrease of cell viability in HT29 cell line, which gave a major contribution to the main goal of the present research, i.e. to determine the dose that might spare the normal tissue. ***Conclusion:*** To our best knowledge, fractionation in several consecutive days in two designed regimes is described for the first time. These are the first reported results using low-doses pre-irradiation followed by hyper and hypo fractionation regimes, with approximately same biological effective dose.

Keywords: *Low dose pre-irradiation, HT29, MRC5.*

INTRODUCTION

The low-dose irradiation (LDI) may have different biological effects in comparison to high -dose irradiation (HDI) [1]. The exposure of cell lines to LDI leads to changes at the molecular level, which may induce adaptive response of cells to ionizing radiation [2, 3]. Based on the functional and single gene investigations, it has been suggested that the adaptive response phenotype is associated with DNA damage repair and stress response functions [2]. Changes of these functions may lead to the reduction of cytogenetic damages, and thus enhance the survival rate in mammalian cells [4-6]. Cells and tissues exposure to low irradiation doses followed by higher irradiation doses is known as the radioadaptive irradiation. The adaptive response can lead to hypersensitivity or radioresistance [7]. Majority of the radioadaptive response experiments are focused on the basic research of this phenomenon, and only a few studies are related to its

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clinical application. The adaptive response could be used in particular for radiotherapy indications. The adaptive dose is low dose which can be applied before one used in irradiation modality, which can be conventional (1.8-2.2 Gy), hyper fractionation (multiple daily fraction) or hypo fractionation (dose per fraction is equal or more than 2.5 Gy). Beside, other radiation modalities exist, but they are not in the focus of present research. Several studies deal with the effect of pre-irradiation doses and variable challenging doses, but exact mechanism of the effect is still unknown [2, 8, 9]. The cell viability is widely used in numerous studies as a parameter to evaluate survival of cells [3]. Studies of a low dose irradiation effects on the cell viability were performed using low doses followed by single high dose irradiation. Schwarz *et al.* [3] showed in HT29 and GM637 adaptive effect suggesting that 0.05 Gy might be the dose, which increases radiosensitivity of the tumor cells with sparing effect on the normal cells. These results induced preparation of our protocol study. Thus, we extended research to MRC5 cells, in addition to the HT29 cell line, and we changed irradiation regimes. Our aim was to increase the response using low doses followed by hyper fractionation and hypo fractionation regimes during 4 days overall treatment time.

MATERIALS AND METHODS

Cell lines

The cell lines used in the study were HT29 (human colorectal adenocarcinoma, American Type Culture Collection HTB-38™) and MRC-5 (human fetal lung fibroblasts, American Type Culture Collection CCL 171). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5% of glucose, supplemented with 10% of fetal calf serum (FCS, NIVNS) and antibiotics: 100 IU/cm^3 of penicillin and 100 mg/cm^3 of streptomycin (ICN Galenika). The cells were sub-cultured twice a week and a single cell suspension was obtained using 0.25% trypsin in EDTA (Serva). All cell lines were cultured in flasks (Costar, 25 cm^2) at 37^{0}C in the 100% humidity atmosphere and 5% of CO_2. Exponentially growing cells were used throughout the assays. The cell density (number of cells per unit volume) and percentage of viable cells were performed as previously described [10]. Viability of cells used in the assay was over 90%.

Irradiation

Both cell lines were irradiated using phantom constructed specially for this experiment. Phantom was made of Plexiglas plates, with four holes to insert the flasks. The phantom was designed based on the experimental requirements, following these principles: to minimize the presence of air between flask and hole where the flask is inserted, and therefore to improve scatter conditions in medium, and to allow isodose coverage 95%-107%. The plexiglas is often used for phantom design, because it shows tissue equivalent characteristics. The size of a phantom was created in order to allow sufficient scatter material around the radiation field, in order to cover the flasks in all three directions, so final dimensions were 30 cm × 30 cm × 5 cm.

Flasks with cell lines were placed into phantom holes, CT scanned, and CT data were imported into the treatment planning system, contoured, and planned with the commercial treatment planning system, Elekta XiO, version 4.62. The cells in experimental samples were pre-irradiated with 0.03 Gy, 0.05 Gy and 0.07 Gy but the control cell samples were not pre-irradiated. Both control and pre-irradiated cells were further irradiated after two hours when hyper and hypo fractionation regimes were applied. Both, hyper and hypo fractionation regimes were approximately calculated based on biologically equivalent dose (BED) of 4 × 2 Gy (conventional regime). For the hyper fractionation the calculated doses were 1.3Gy [twice per day with four hours period between daily fractions [11, 12]] during three consecutive days. For the hypo fractionation, the calculated doses were 4.6 Gy (once per day) on the first and fourth day, in order to obtain same overall treatment time as in hyper fractionation regime. The described regimes of irradiation were repeated twice.

The determined irradiation doses were estimated as biological equivalent (BED) to four-day treatment with 2 Gy fraction (conventional

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regime). The given doses were calculated according to the radiobiological formula:

Basic equations for BED:

$$E= n(\alpha d+\beta d2)=D(\alpha+\beta d)$$

E-effectiveness; n-number of fraction; d-dose per fraction; D-total dose; α, β – proportional coefficients

$$SF=\exp(-E)=\exp[-(\alpha+\beta d)D]$$

SF=survival fraction

$$D/Dref=dref+(\alpha/\beta)/d+(\alpha/\beta)$$

$$EQD =d+(\alpha/\beta)/2+(\alpha/\beta)$$ [13]

Colorimetric MTT (tetrazolium) assay

The cell viability was evaluated by tetrazolium colorimetric MTT assay (SIGMA). The assay is based on the cleavage of the tetrazolium salt MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), to formazan by mitochondrial dehydrogenases in viable cells [14]. MTT (5 mg/ml) was dissolved in phosphate buffered saline (PBS) and filtered to sterilize and remove a small amount of the insoluble residue present in some batches of MTT. Stock MTT solution (10µl per 100µl medium) was added to all wells of an assay, and plates were incubated at 37°C for 4h. Acid isopropranol (100µl of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a spectrophotometer microplate reader (Multiscan MCC340, LabSystems) at 540/690 nm. Plates were normally read within 1h of adding the isopropanol. The wells without cells containing complete medium and MTT only acted as blank.

Data analysis

All continuous variables are expressed as means ± standard deviation (SD). The differences in average values were determined using the Tuckey test in STATISTICA, version 10.0 [15]. Probability values of less than 0.05 were considered as statistically significant.

RESULTS

The absolute number of metabolically active cells in two cell lines after applied irradiation modalities is presented in table 1. Differences in the means of cell viability obtained by MTT assay were determined within each irradiation modality. The differences were analyzed separately for the each cell line.

Radio-adaptive doses effects on HT29 cell line were as follows: pre-irradiation dose of 0.05 Gy in hyper fractionation regime and control without pre-irradiation expressed statistically significant decrease of the cell viability ($p<0.05$), compared to the pre-irradiation doses of 0.03 Gy and 0.07 Gy. In hypo fractionation regime 0.05 Gy and 0.07 Gy expressed statistically significant decrease of the cell viability ($p<0.05$), compared to control without pre-irradiation.

In hyper fractionation regime, the pre-irradiation dose of 0.05 Gy expressed statistically significant increase of the cell viability ($p<0.05$), in MRC5 cell line compared to both others pre-irradiation doses, as well as with the control without pre-irradiation. In hypo fractionation regime in the same cell line the pre-irradiation dose of 0.05 Gy expressed increase, although non-significant, comparing to the control without pre-irradiation and 0.07 Gy, and statistically significant increase was registered comparing to 0.03 Gy pre-irradiation dose.

Comparison of the responses of two cell lines in the same regime revealed that the pre-irradiation dose of 0.05 Gy induces increase of cell viability in MRC5 cell line (not always statistically significant, but present), accompanied with the decrease of cell viability in HT29 cell line, which gave a major contribution to the main goal of the present research (figure 1 and figure 2).

DISCUSSION

The goal of radiotherapy is to improve therapeutic tumor control using different modern approaches and at the same time to spare surrounding tissues [3]. The number of research on the field of radiation oncology focus on determination of optimal radiotherapy treatment, regarding applied doses and fractionation,

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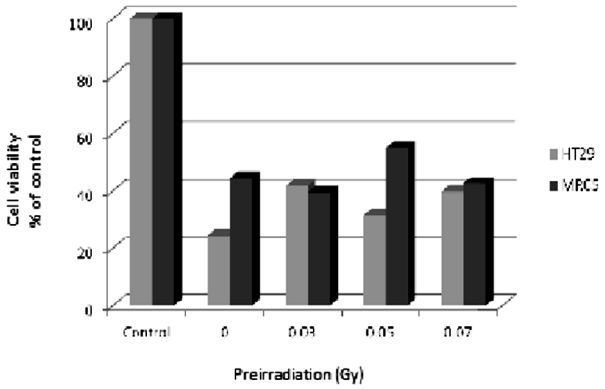

Figure 1. Relative ratio of cell viability after different pre-irradiation doses in hyper fractionation regime.

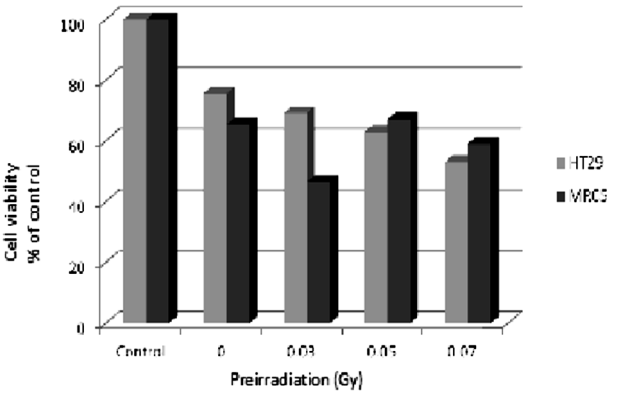

Figure 2. Relative ratio of cell viability after different pre-irradiation doses in hypo fractionation regime.

in order to minimize side effects on normal tissues [1]. In this study, low-doses pre-irradiation followed by different irradiation regimes in several days led to the radioadaptive response of tumor HT29 cell lines, and normal MRC5 cells, as was expected, eventhough some new interesting results were obtained.

Significant decrease in the cell viability and metabolic activity in colorectal cancer cells HT29 irradiated in hyper fractionation regime with and without pre-irradiation doses was found compared to non-irradiated control. The pre-irradiation doses of 0.05 Gy and non pre-irradiated control in the same regime induced more pronounced, statistically significant, decrease in viable HT29 cells compared to pre-irradiation cells with 0.03 and 0.07 Gy priming doses (table 1, figure 1). It might be concluded that doses of 0.03 Gy and 0.07 Gy in hyper fractionation regime decreased apoptotic effect. It is known that in mammalian culture systems, pre-irradiation with 0.02 Gy of X-rays 5 h before the second exposure significantly enhanced the survival rate and produced a reduction in the number of chromosomal aberrations [16]. Our results are similar to results of Lambin *et al.* [17] who reported on increased X-ray sensitivity of HT29 cell line, with single-doses of X-rays from 0.05 Gy to 5 Gy. Lambin *et al.* [17] focused on cell survival at doses less than 1 Gy, and showed sensitivity at doses less than 0.5 Gy. In addition, Schaffer *et al.* [1,19] found radiosensitive effect of

Table 1. The mean absolute number of metabolically active cells in two cell lines after applied irradiation modality.

Fractionation regime	Applied doses	Cell number x10^6±SD	
		HT29	MRC5
Control	Nonirradiated cells	0.734 ±0.045	0.407± 0.027
	Preirradiation		
Hyper fractionation	0 Gy	0.179± 0.028*	0.181±0.011
	0.03 Gy	0.305±0.030	0.160±0.005
	0.05 Gy	0.231±0.025*	0.223±0.027*
	0.07 Gy	0.291±0.031	0.173±0.011
Control	Nonirradiated cells	0.323±0.038	0.222±0.035
	Preirradiation		
Hypo fractionation	0 Gy	0.244±0.019	0.145±0.004
	0.03 Gy	0.223±0.026	0.103±0.008
	0.05 Gy	0.203±0.019*	0.149±0.005
	0.07 Gy	0.171±0.006*	0.131±0.005

*p<0.05; Tuckey test

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priming dose of 0.05 Gy. In our research in hypo fractionation regime, significant decrease of cell viability in HT29 cell line was registered after 0.05 and 0.07 Gy pre-irradiation doses compared to non-preirradiated control. These findings are similar to previously published results on 0.05 Gy priming dose followed by single challenging dose [1,3,19].

Regarding the effects of low-dose pre-irradiation on lung fibroblasts cells MRC5, we found spearing effect for some priming doses. In hyper fractionation, 0.05 Gy led to significant increase of metabolic activity compared to non-preirradiated control. In hypo fractionation regime increase of the cell viability was observed after priming dose of 0.05 Gy compared to non-preirradiated control, even though not statistically significant. Therefore, in both applied regimes, priming dose of 0.05 Gy enhanced cell viability, comparing to other priming doses and non-preirradiated control.

Thus, our main goal to determine the dose that might spare the normal tissue was detected. The encouraging fact was that dose of 0.05 Gy at the same regime, caused significant decrease of cell viability on HT29 cells while increased metabolic activity of MRC5 cells. In addition, this 0.05 Gy prior to the 2.0 Gy fraction [3] showed enhanced mortality of colorectal cancer cells without damage of normal fibroblasts. Our results were similar to regimes in radiotherapy, eventhough we used different regimes applied during four days, which gave additional significant point compared to just one fraction which was uncommon approach in clinical practice. Altered fractionated regimes led to better locoregional tumour control, compared to 2 Gy conventional fractionation regime, evethough can lead to more acute toxicity for normal tissue [20]. In this study, we have tried to apply hyper and hypofractionated regimes in the same overall tretment time, since they are less aplicable in radiotherapy, and we wanted to evaluate and to improve their potential by low-dose pre-irradiation. Our results confirmed that 0.05 Gy pre-irradiation dose may play important role in normal cells sparing (MRC5 cells), with significant decrease of cell viability of tumor cells (HT29 cells). The research on cell lines using more fractions gave more valuable results compared to investigations of radioadaptive responses after single dose irradiation [21].

A possible way to explain the effects of 0.05 Gy pre-irradiation dose would be investigation of the gene expression for the components that are involved in the double-brake strand DNA repair systems [18]. Basic researches in radiobiology investigate the effects of different irradiation doses and modalities of delivery but exact mechanism of the observed effects is still unknown [19]. It is widely accepted that altered gene expression is caused by low-dose ionizing radiation. It seems that radio-adaptive response is associated with an up-regulation of DNA repair and stress response genes and by down-regulation of cell cycle control and apoptosis genes [18, 19]. In order to fully explain observed low-dose pre-irradiation effects, we are going to continue our study at the molecular level evaluating molecular mechanisms, which underlie the detected responses.

Comparing the observed effects of hyper and hypo fractionation regimes combined with low-dose pre-irradiation, it can be concluded that pre-irradiation dose of 0.05 Gy in both regimes led to spearing of MRC5 cells. This priming dose could be the dose of choice to improve the benefit of therapeutic ratio according to our results obtained 72h after the end of irradiation regimes. The pre-irradiation dose of 0.05 Gy gave the most valuable effect and clinical evaluation of these findings (not only ours [1, 3, 19]) is worth to be performed in the future.

It is important to stress out the fact that as far as we know, all previous researches on low-dose pre-irradiation effects were done with different cell lines using several low-doses followed by single irradiation therapeutic dose. To our best knowledge, fractionation in several consecutive days in two designed regimes was described for the first time.

These are the first reported results using low-doses pre-irradiation followed by (a) 1.3 Gy dose twice per day or (b) with 4.6Gy on the first and the fourth day. In each regime, additional dose was applied two hours after pre-irradiation.

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CONCLUSION

Cell viability can be modified using low pre-irradiation doses followed by fractionation regime or a single challenging dose. In the present study, we showed effects of radio-adaptive doses on HT29 and MRC5 cells. Low-dose pre-irradiation, followed by hyper or hypo fractionation regimes, seems to have better influence to radiosensitivity or radioresistance compared to low-dose pre-irradiation followed by a single dose. Obtained results suggested that pre-irradiation low dose of 0.05 Gy caused significant decrease of HT29 cell viability while significantly increased the number of MRC5 cells which indicated enhanced toxicity to colorectal cancer cells without damage to normal fibroblasts.

Conflict of interest: *Declared none*

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