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Changes in cellular response to the damage induced in PC-3 prostate cancer cells by proton microbeam irradiation

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Abstract. The aim of this research was to find out whether the passage number effect may influence on the PC-3 cells (the human prostate cancer line derived from bone metastases) response to proton radiation. 2 MeV horizontally focused proton microbeam was used as a radiation source. The cells were treated with a counted number of H^+ ions (50–8000) corresponding to doses of 1.3–209 Gy/cell. For comparison, cell death was also induced by UVC radiation. All cells were stained with Hoechst 33342 and propidium iodide and visualized under a fluorescence microscope. Necrosis was observed at: a) 8000 protons *per* cell (corresponding to ~209 Gy/cell) after 2–4 passages, b) 3200 protons *per* cell (corresponding to ~84 Gy/cell) for cells after 11–14 passages and c) only 800 protons *per* cell (corresponding to ~2 Gy/cell) after 47–50 passages. Apoptosis was efficiently induced, by protons, only in cells after 50 passages. The results showed that the laboratory conditions affected cellular response of PC-3 cell line to the proton irradiation. The cellular response to the radiation treatment strongly depends on number of passages.

Key words: PC-3 cells — Single cell irradiation — Cellular response — Proton microprobe

Introduction

Cancer cell lines are commonly used as a model in scientific research because of their immortality and also phenotypic and genotypic stability up to tens of passages. The cell cultures provide relatively simple and reproducible experimental models. They allow examining a particular type of cells and studying many biological processes such as proliferation, cell survival, susceptibility to cyto- and genotoxicity factors, DNA repair capacity (rev. Langdon 2004). However, an increasing number of publications demonstrate that cell culturing can change the properties of the cell lines. Various types of changes in phenotype, genetic modifications, proteins and gene expression were observed with increased number of passages (Briske-Anderson et al. 1997; Chang-Liu and Woloschak 1997; Esquenet et al. 1997; Wegner et al. 2004; Sambuy et al. 2005). Modification of cells observed with increasing number of passages has been called *passage number effect*. The knowledge about this phenomenon is not fully explored because it strongly depends on host factors, such as the tissue species origin, the cell line type and the culture conditions.

One of the research focuses in radiobiology is estimation of the biological effectiveness of different radiation qualities. The most important fields are: the risk evaluation on the health in the environmental study, and also maximization of the beneficial effect in tumor radiotherapy (Folkard et al. 2005; Gerardi 2009). For several years, there are new opportunities for studying key mechanisms of ionizing radiation: single-particle microbeam facilities. They are excellent tools applied in radiation biology research (Folkard et al. 2005; Gerardi 2009). They provide the possibility of targeting individual cells within a population with defined numbers of particles. In this study, the proton microbeam was applied as a proton source. In the Institute of Nuclear Physics (Kraków) a microbeam setup based on the Van de Graaff accelerator was designed and constructed (Polak et al. 2006a,b; Veselov et al. 2006). This microbeam facility allows to irradiate single cells with a specific number of protons. It gives us possibility to conduct a study of the biological effect of proton irradiation.

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The aim of this study was to find out whether the passage number effect may influence the PC-3 cells response to proton radiation. In a comparison study, UVC was applied as another radiation source. In the presented research the human prostate adenocarcinoma derived from bone metastases – PC-3 cell line was used. This cell line is often used as a model in prostate cancer study. The high degree of invasiveness of PC-3 line makes it a very interesting research model. PC-3 cells are p53-null, they are androgen independent and poorly differentiated (Kaighan et al. 1979; Alimiraha et al. 2006). The changes in response of cells kept in laboratory conditions from a few to tens of weeks were examined. The viability of cells exposed to radiation was investigated. Morphological features of necrotic and apoptotic cells were detected and counted.

Cell death plays an important role in physiological and pathological processes including development, aging, and disease. From many cases of cell death, the research encompasses not only the study of programmed cell death (apoptosis, autophagic cell death) but also necrosis and other modes of cellular demise (Degterev et al. 2008; Fulda et al. 2010). Necrosis possesses characteristic features, such as organelle swelling, mitochondrial dysfunction, massive oxidative stress and rapid plasma-membrane permeabilization that are thought to be indicative of the catastrophic nature of cell death rather than a result of cellular regulation (Degterev et al. 2008; Alvarez et al. 2010). However, there is now growing evidence that the execution of necrotic cell death is also regulated by a set of signaling pathways (Krysko 2006; Berghe et al. 2010; Poon et al. 2010). Necrosis as well as necroptosis, and secondary necrosis following apoptosis represent different modes of cell death that result in similar cellular morphology (Berghe et al. 2010; Poon et al. 2010). They differ in the kinetics of cellular disintegration. For example, $H_2O_2^-$ induced necrosis, due to presence of reactive oxygen species (ROS), starts immediately by lysosomal permeabilization (Berghe et al. 2010). In contrast, TNF-mediated necroptosis and anti-Fas-induced secondary necrosis need more time which results from the mobilization of signaling pathways (Poon et al. 2010; Krysko 2006). Necrosis induced by irradiation could be an instant process. In direct deposition of energy or by reactive oxygen species (ROS), ionizing radiation damages the cell membrane which causes the culture medium to flow through the cell membrane inside the cell. The cellular radiosusceptibility changes, seen as increasing necrosis with increasing passage number, might be the consequence of the cell membrane modification (which means that the cell's phenotype was changed during culturing).

Apoptosis is a complex, controlled, and programmed process characterized by a caspase-dependent signaling phase. Increasing or decreasing of apoptosis processes depends on the gene expression and is connected with modification of cellular genotype. Apoptotic cells contain morphological features, such as nuclear fragmentation, membrane blebbing and the formation of apoptotic bodies that can be used to identify apoptotic cell death events (Degterev et al. 2008; Fulda et al. 2010).

Materials and Methods

Cell culturing

PC-3 cells (human prostate adenocarcinoma derived from bone metastases) were cultured in flasks (25 cm^2 growth area), as mono layers in RPMI 1640 medium, supplemented with 10% of FCS (fetal calf serum), 100 U/ml penicillin-streptomycin solution, 10 mM HEPES, 1 mM sodium pyruvate and 4.5 mg/ml glucose (all compounds delivered by Sigma Aldrich, Stenheim, Germany). Cell culture was conducted in 37°C and the atmosphere of 5% CO_2. 0.5% trypsin in PBS (phosphate buffered saline) was used to collect cells. Cell culturing procedure was accurately described in Podgórczyk et al. (2009). Cells were passaged once a week. For the purpose of the study the cultures were carried out continuously until cells with high passage number were obtained. Irradiation procedure was performed when cells were characterized by appropriate passage number – cells after 1–3, 12–14, and 47–50 passages.

Irradiation sources

2 MeV horizontal focused proton microbeam (the external beam diameter of about 16 μm at the irradiated spot) from the Van de Graaff accelerator was used as a proton source. During cell irradiation the beams' current was about 0.16 fA, which corresponds to 1000 protons *per* s. The targeting accuracy was 92%, meaning that 92% of protons were located within 30 μm spot (Polak et al. 2006a,b). Microprobe raster scanning mode of operation was used, where the given number of protons *per* 20 μm beam step was applied. The scan step size was comparable to the size of the PC-3 cells. Therefore, the number of protons *per* 20 μm beam step was corresponding to the number of protons *per* one cell. The number of protons was counted by a silicon surface barrier particle detector (Ortec B-019-300-150). This detector was used to measure the energy deposited in one cell.

Doses were calculated based on definition of the dose $d = \bar{E}/m$; where $\bar{E}$ is averaged energy deposited by proton beam in the cell ($\bar{E}$ = LET h; LET = 16 keV/μm counted by SRIM) and m is cell mass.

The cell size needed to mass calculation was calculated by using model: $V = abh/12$; where a = 25 μm, b = 15 μm, h = 12 μm – taken as an average elongate (spindle) cell size.

In a comparison study, the ultraviolet C (UVC; wavelength 254 nm) radiation was used. The radiation source

was a laboratory UV lamp. The density of energy was about 24 mW/cm^2, which is about 200-times greater than density of radiation energy used for sterilization in laminar flow chamber at the level of the investigators hands. This density of energy was chosen to induce necrosis in less than 20 min.

Cell irradiation

For proton irradiation cells were seeded on specially prepared 35 mm diameter Petri dishes, in which 10 mm round holes were cut in the central part of the bottom. The Petri dish bottom was covered with the 1.5 μm thick Mylar foil (Goofellow Cambridge Limited, Huntington, UK) using the glue (Master Bond EP 30 med, New York, USA). A population of about 10^5 cells in 4 μl medium was seeded on the central part of the Mylar foil 16–18 hours before the experiments. Four hours after the seeding, when the cells had adhered to the foil, 2 ml of medium was added.

Single cells were targeted with a number of counted H^+ ions, from 50 to 8000, corresponding to the dose between 1.3 Gy and 209 Gy *per* cell. Proton irradiation of cells was in details described (Polak et al. 2006a,b; Veselov et al. 2006; Ugenskiene et al. 2007). Irradiation of cells growing on one dish did not take more than 20 minutes (usual time needed for cell passaging). In each experiment 500 to 1000 cells were hit with the same chosen number of protons. The experiment was repeated at least ten times, for each treatment point.

In parallel experiments, cell death was induced by UVC radiation. The UVC irradiation was carried out in a laminar chamber in sterile conditions. The cells were irradiated for 0 to 20 minutes, at a distance of 25 mm from the light source. The cells were seeded on the Petri dishes, as in the main experiment without modifications.

Control cells for each kind of irradiation source were grown on the separated dishes and the same procedure of seeding was followed like in case of treated cells.

Microscopic analysis

The untreated and irradiated cells were observed under fluorescent microscope (Olympus BX51) 20 hours after irradiation. Two fluorescent dyes: Hoechst 33342 (10 μg/ml) first and when all cells were stained in blue, propidium iodide (PI, 1 μg/ml) were added. The staining procedure was performed 15 min *prior* to microscopic evaluation to avoid increased death cells due to fluorescent dyes.

Both fluorochromes are agents intercalating to nucleic acids. Necrotic cells are PI positive. When the cell membrane is damaged, PI leaks into the cell and binds to DNA and RNA resulting in red fluorescence of necrotic cells. All blue cells are Hoechst positive; the dye was applied to identify cells with

Table 1. Percentage of dead (apoptotic and necrotic) and alive cells after proton irradiation

Treatment		Apoptotic cells (%)			Necrotic cells (%)			Living cells (%)		
No. of passage		2–4	11–14	47–50	2–4	11–14	47–50	2–4	11–14	47–50
Untreated cells		0.29 ± 0.66	2.85 ± 0.02	0.02 ± 0.01	0.28 ± 0.43	0.15 ± 1.88	0.11 ± 0.23	99.43 ± 0.79	97.00 ± 1.89	99.87 ± 0.23
Protons/cell	50	–	–	93.67 ± 2.90	–	–	0.40 ± 0.13	–	–	5.93 ± 2.91
	200	1.24* ± 1.94	1.5* ± 3.1	100.00* ± 0.00	3.05 ± 0.02	1.74 ± 2.00	0.00 ± 0.00	95.71* ± 1.94	96.76* ± 3.68	0.00* ± 0.00
	400	3.79* ± 0.95	1.31* ± 0.92	100.00* ± 0.00	2.78 ± 3.37	3.51 ± 1.18	0.00 ± 0.00	93.43* ± 3.50	95.18* ± 1.50	0.00* ± 0.00
	800	0.37* ± 0.64	1.41* ± 1.1	0.83* ± 0.58	7.35 ± 0.8	3.21 ± 0.20	98.90 ± 4.70	92.29* ± 1.02	95.38* ± 1.12	0.27* ± 4.73
	1600	0.9 ± 2.85	1.62 ± 1.11	–	4.53 ± 0.01	5.38 ± 2.00	–	94.57 ± 2.85	93.00 ± 2.28	–
	2400	0.29 ± 0.66	1.01 ± 1.10	–	4.85 ± 2.06	4.69 ± 0.40	–	94.86 ± 2.16	94.30 ± 1.17	–
	3200	1.75 ± 2.08	1.92 ± 1.31	–	5.67* ± 1.35	59.89* ± 17.00	–	92.57* ± 2.48	38.19* ± 17.04	–
	4800	5.3 ± 1.53	–	–	0.99 ± 2.02	–	–	93.71 ± 2.53	–	–
	8000	6.5 ± 0.78	–	–	66.93 ± 7.05	–	–	26.57 ± 7.09	–	–

*Significant differences between cells from various passage numbers; $p < 0.05$. Data were expresed as mean ± SEM.

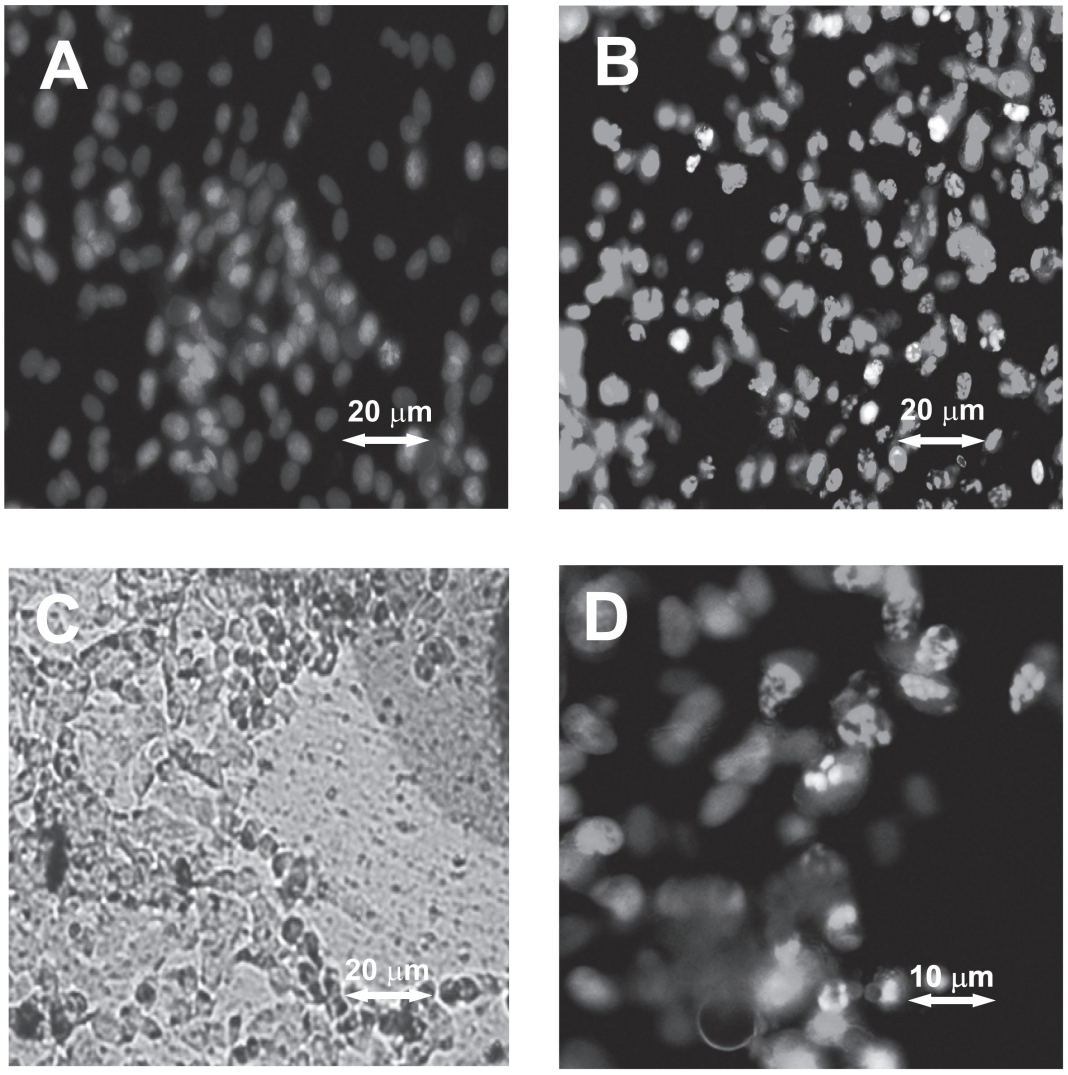

Figure 1. PC3 cells (48th passage) response to the proton radiation (staining by PI and Hoechst): control PC-3 cell nuclei (**A**), apoptotic cell nuclei irradiated by 50 protons *per* cell (**B**), cells irradiated by 200 protons *per* cell, area without cells and control cell nuclei (**C**) and apoptotic PC-3 cell irradiated by 200 protons *per* cell (**D**; edge of the area from which the cells came unhit).

nuclear fragmentations (morfological features of apoptosis) and living cells (characterized by a presence of one nucleus). When cells were PI positive and additionally possessed nuclei damages (probably cells in late stage of apoptosis, secondary necrosis), they were counted as apoptotic ones. It occurred only in cells after 47–50 passages. Image Pro Plus 6.0 free software (Media Cybernetics) was used to automatically count the red (PI positive) and the blue (Hoechst positive) cell nuclei. Thousand cells *per* treatment were analyzed. When the cells detached 20 hours after irradiation, they were considered as apoptotic ones. Then the detached cells were counted as difference between the number of exposed and the number of attached cells.

Results

Dose-response relationship

The cell response on the proton radiation was presented in Table 1. For all passages, the lived cells (after irradiation) decreased with increasing number of protons. Two different cell death cases were detected and counted according to morphological criteria as described above. In our study, necrosis as a cell death was more frequent as compared to apoptosis. However, a ratio of both types of death depended on the proton dose. For example: in "the youngest" cells: 200 protons *per* cell cause 1.24% apoptosis and 3.05% necrosis,

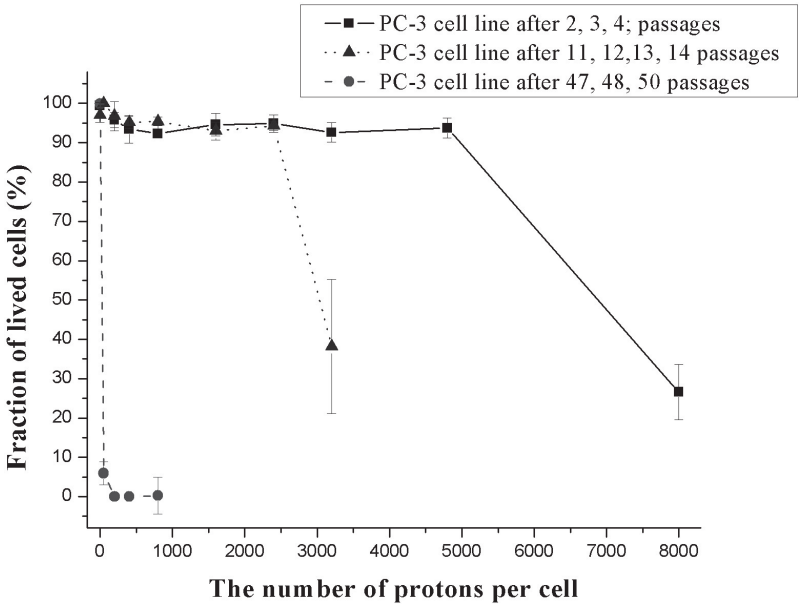

Figure 2. The fraction of alive cells (from the different passage number) as a function of number of protons *per* cell.

400 protons *per* cell cause 3.79% apoptosis and 2.78% necrosis (Table 1). The effect of different dose on the response of cells from 47th passage is also presented in Fig. 1.

Effect of the passage number

The number of irradiated and alive cells dependend on passage number: the higher pasage number, the lower amount of living cells (Fig. 2). Necrosis was observed at 8000 protons *per* cell (corresponding to ~209 Gy/cell) after 2–4 passages, 3200 protons *per* cell (corresponding to ~84 Gy/cell) for cells after 11–14 passages and only 800 protons *per* cell (corresponding to ~21 Gy/cell) after 47–50 passages. Apoptosis was detected only in cells after 47th passage and older ones (Table 1). This type of cell death occurred after irradiation by 50, 200, 400 protons *per* cell (corresponding to ~1.3, ~5.2, ~10.4 Gy dose). Moreover,

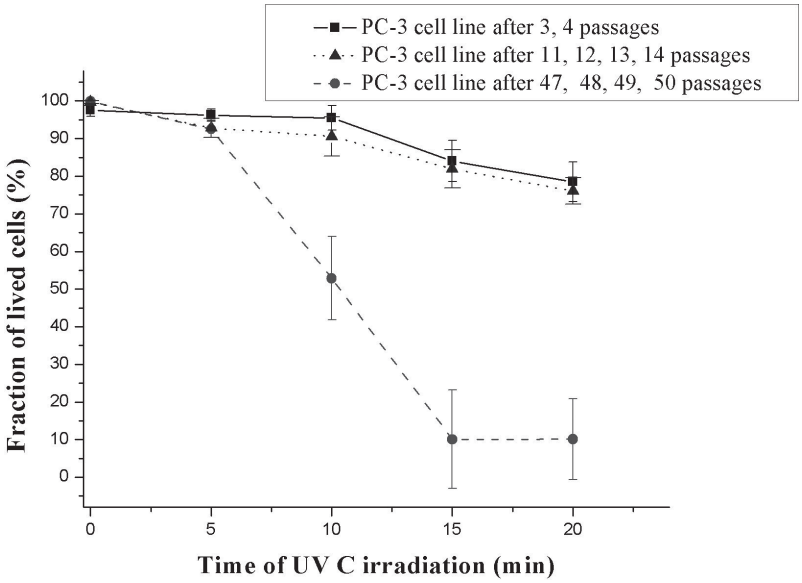

Figure 3. The fraction of alive cells (from the different passage number) as a function of UVC radiation time.

Table 2. Percentage of dead (apoptotic and necrotic) and alive cells after UVC irradiation

Treatment		Apoptotic cells (%)			Necrotic cells (%)			Living cells (%)		
No. of passage		2–4	11–14	47–50	2–4	11–14	47–50	2–4	11–14	47–50
Untreated cells		0.29 ± 0.66	2.85 ± 0.02	0.02 ± 0.01	0.28 ± 0.43	0.15 ± 1.88	0.11 ± 0.23	99.43 ± 0.79	97.00 ± 1.89	99.87 ± 0.23
UVC (min)	5	0.37 ± 0.64	0.10 ± 0.22	0.20 ± 0.41	3.40 ± 1.50	7.08 ± 2.53	7.27 ± 2.22	96.24 ± 1.63	92.82 ± 2.54	92.53 ± 2.26
	10	0.90 ± 2.85	0.20 ± 0.28	0.09 ± 0.31	3.62* ± 1.50	9.22* ± 5.21	47.06* ± 11.06	95.48* ± 3.22	90.58* ± 5.21	52.85* ± 11.06
	15	0.29 ± 0.66	0.13 ± 0.23	0.11 ± 0.32	15.69* ± 5.40	17.92* ± 5.04	89.81* ± 13.13	84.02* ± 5.44	81.95* ± 5.04	10.08* ± 13.13
	20	0.38 ± 0.27	0.07 ± 0.14	0.19 ± 0.41	21.09* ± 5.30	23.85* ± 3.55	89.69* ± 10.78	78.54* ± 5.31	76.09* ± 3.55	10.12* ± 10.79

*Significant differences between cells from various passage numbers; $p < 0.05$. Data were expresed as mean ± SEM.

all cells from 47th passage irradiated by 200 and 400 protons *per* cell (corresponding to ~5.2 Gy and ~10.4 Gy dose) were detached (Fig. 1C). This irradiation treatment was repeated ten times with the same result. The finding is in agreement with other studies. Modification of cell adhesion is a well known effect of apoptosis (Suzanne et al. 2009). Therefore detachment suggests that apoptosis progressed faster in cells irradiated by 200 and 400 protons *per* cell than in cells irradiated by 50 protons. Mortality of high passage cells after proton treatment was higher in comparison to mortality of low passage cells (ANOVA test: the 5% significance level) (Table 1).

UVC irradiation study

Complementary to the proton irradiation study, the experiments with UVC as an irradiation source were made. The survival curves for cells after 1–3 passages and 11–14 passages were comparable, while for "the oldest" cells significant decrease of cell survival was observed (Fig. 3). Mortality of the high passage cells after UVC radiation, as well as after proton treatment was higher in comparison to mortality of the low passage cells (ANOVA test: the 5% significance level) (Table 2). Necrosis in the PC–3 cell line occurred after 5 min of UVC irradiation and increased with the time of UVC exposure as well as with the passage number (Table 2). All attempts to induce apoptosis in PC-3 cells by UVC irradiation failed. The number of cells with nuclei fragmentation was similar to the control (Table 2).

Discussion

In the presented research, PC-3 cells were treated by protons and also UVC. The cell response to the radiation as a function of cell passage number was studied. It was found that PC-3 cell response to the radiation strongly depends on the passage number of cells cultured in laboratory conditions. In particular the passage number effect observed here has already been investigated in several publications. For example, pancreatic beta cell line MIN6 long-term culture was found to be associated with many phenotypic changes, including changes in growth rate and cellular morphology (O'Driscoll et al 2006). Caco 2 (cell line from human colon adenocarcinoma) after 72nd passages and long culturing time (Day 21), had significantly lower alkaline phosphatase activity than did the other passages (Briske-Anderson et al. 1997). Additionally, the some of cell areas were not monolayers; cells preferred multilayer formation (Briske-Anderson et al. 1997). Apart from phenotypic changes various types of genetic modifications and gene expression were also observed. High-passage breast cancer cell line MCF-7 exhibits a decrease in GFP (green fluorescent protein) levels (Wegner et al. 2004), while high-passage Caco-2 cells mentioned above, show an

increase in the expression of GFP reporter gene after transfection (Sambuy et al. 2005; Hughes et al. 2007).

In case of the prostate cancer, LNCaP line (androgen-sensitive human prostate adenocarcinoma cells derived from the supraclavicular lymph node metastasis) proteomic characteristics of low (L-33) and high (H-81) passage numbers showed significant differences in protein expression (Youm et al. 2008). Authors identified five proteins (Tim, cathepsin D, CKB, GRP78, HSP27) that exhibited significantly different changes between the low and high passage number. Other authors (Esquenet et al. 1997) reported changes in LNCaP cell line response to the synthetic androgen in cells from various passage numbers. Alimirah and co-authors (Alimirah et al. 2006) found the androgen receptor mRNA and protein in PC-3 cells, which are used in the present study. Although the level of androgen receptor protein in PC-3 cells was lower than in LNCaP cells, it is possible that passage number depended- response to the androgen might also be considered in PC-3 cells. In particular, Heisler and co-authors reported androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell after androgen receptor transfection (Heisler et al. 1997). Thus, passage number effect might induce changes resulting in growth inhibition and apoptosis. That is consisted with our findings. We observed changes in cell response to induction of cell death by radiation in dependence on the passage number.

In the presented results PC-3 cells response to the radiation is observed, expressed as higher percent of dead cells after irradiation with the increased number of passages. It could be a consequence of changes in cell phenotype. Moreover, activity of mechanisms protecting cells against apoptosis decreased with the increased of culturing time. That might be a result of changes in protein and gene expression (Chang-Liu et al. 1997; Wegner et al. 2004).

Effect of the passage number on cellular response to ionizing radiation was also reported by Chang-Liu and Woloschak (1997). Authors observed that increased passage number, Syrian hamster embryo (SHE) cells demonstrated decreased doubling time, increased plating efficiency, and decreased the number of cells *per* plate. However, these changes were accompanied with changes in control, gamma-ray and UV-induced gene expression. According to authors ionizing radiation sensitivities did not change with passage number; the changes in gene expression were evident in gamma-ray exposed cells as well as in the control. In contrary, in our research we did not observed increasing morphological features (such as nucleus fragmentation, cell membrane damage) with increasing passage number in control cells. The changes concerned only cells after irradiation. That is why we assumed that passage number effect influences the PC-3 cell line response to proton and UVC irradiation. Moreover, the passage number effect was critical in experiments conducted by Belyaev and co-authors (Belyaev et al. 1996). Authors studied effect of the gamma-rays on chromatin conformation in normal human VH-10 diploid fibroblasts. They received uniform and reproducible results at passage numbers: 12–14. The increase at 10% of cell viscosity (correlated with chromatin conformation) was detected already at the 18 passage.

We conclude that changes in cellular response to the damage induced in PC-3 prostate cancer cells by proton microbeam irradiation are associated with phenotypic and genotypic modifications accumulated during the culturing. These changes appear in cells already after only several passages. These results emphasize the inherent variability in PC-3 cell models and emphasize the need to monitor closely the culture characteristics during growth and differentiation under specific experimental conditions. The study of response to the irradiation implies a need of the strict and severe procedures during results interpretation.

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