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CELL DEATH IN IRRADIATED PROSTATE CANCER CELLS ASSESSED BY FLOW CYTOMETRY

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Summary: Despite the significant advances in cancer chemotherapy, radiotherapy still remains a method of choice for treatment of metastatic human prostate cancer. This study presents quantitative analysis of ^{60}Co gamma-radiation effects on cell growth and cell death of metastatic human prostate cancer PC-3 cell line, performed in time (24–72h) and dose (2–20 Gy) dependent manner. The irradiated PC-3 cells were mostly dying by necrosis at late time intervals (72h), while apoptotic cell death was negligible. The EC_{50} or 50% of cytotoxicity was not achieved within the radiation doses used (2–20 Gy), but significant cell growth inhibition with IC_{50} of 10.4 Gy was observed. It is concluded that the increase in the radiation dose may have an important cytostatic effect, but for the complete eradication of metastatic prostate cancer novel cytotoxic drugs and radiosensitizers should be introduced as adjuvant.

Key words: human prostate cancer, cell death, gamma-rays, flow-cytometry

Introduction

Despite the significant advances in the area of cancer chemotherapy, radiotherapy, applied either alone or as adjuvant, still remains a method of choice for treatment of many malignant diseases. One of them is prostate cancer, the most frequent cancer in men population, which unfortunately shows a continual casualty increase (1). The ultimate aim of radiotherapy is to efficiently eradicate tumor cells with minimal deleterious effects to the surrounding normal tissues and to the whole organism. In that view, induction of apoptosis is very desirable therapeutic endpoint (2, 3). However, much is yet to be learned about either systemic or individual biological effects of both conventional (gamma-, x-ray) or accelerated particle (proton, etc.) ionizing radiation in order to optimize clinical results of treatment of human prostate cancer.

Regardless of the routine use of simple test for prostate specific antigen in sera, that can detect disease at an early stage (4, 5), the number of men with metastatic prostate cancer is still high. The early stages of disease are usually managed by ionizing radiation and/or hormone therapy, but there is no successful therapy for metastatic prostate carcinoma. Advanced disease is mostly treated by radiation therapy, sometimes in combination with hormone or chemotherapy, but hormone withdrawal often leads to selection of hormone-independent clones (6). Dose-escalated (*i.e.* 70–80 Gy) radiotherapy is an important treatment option especially for men with intermediate-risk prostate cancer (7). On the other hand, radiotherapy is often inefficient due to radioresistance of prostate cancer cells.

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Recent studies suggest that some prostate cancer cells can undergo apoptosis (8). The response to ionizing radiation, depends on a number of factors such as the stage of differentiation, mutations in specific genes (such as p-53 and bcl-2) that will determine the ability of the target cells to enter apoptosis (9, 10). For clinical purposes (*i.e.* eradication of the tumor, but prevention of undesired inflammatory *sequelae*, radiation sickness and fibrosis), it is useful to investigate whether the cells of certain types are susceptible to apoptosis or necrosis, as well as to determine the time and dose dependence of the process. In addition to cell killing, radiation can also lead to cell cycle arrest and stopping of proliferation with significant decrease in cell growth (11).

The purpose of this study is to investigate radiation induced cell death in PC-3 prostate cancer cell line in time- and dose-dependent manner. The changes in cell growth following irradiation were also determined.

Materials and Methods

Cell lines. Human prostate cancer cell line PC-3 was purchased from American Type Culture Collection (CRL 1435, Rockville, MD). They are androgen independent and were established from bone metastasis, which is the most usual place for metastatic prostate cancer. PC-3 were maintained in RPMI 1640 medium supplemented by 10% heat inactivated fetal calf serum, 100 IU/mL penicillin/streptomycin and 2 mmol/L L-glutamine (Sigma Aldrich Chemie GmbH, Germany), at 37 °C under 5% CO_2 atmosphere. Cells were grown as monolayers in 75 cm^2 culture bottles supplied with 15 mL RPMI, and after a few passages cells were transferred in 25 cm^2 culture bottles (Nunk, Nalgene, Danmark).

Cell Irradiation. For investigation of radiation induced effects on PC-3, 3×10^5 cells were seeded in 25 cm^2 culture flasks, and after 72 hours were irradiated at room temperature with 2, 10 or 20 Gy gamma-rays from ^{60}Co gamma-source, at the dose rate of 20 Gy/h. The effects of irradiation on cell viability, morphology and genomic DNA structure were determined 24-, 48-, and 72 h after irradiation.

Trypan blue exclusion assay. For analysis of cell growth and spontaneous cell death in culture, cells were seeded at a density of 12×10^3 cells/cm^2 in 25 cm^2 culture flasks. Cell growth, viability and morphology were followed for 8 consecutive days, by trypan blue exclusion (TBE) assay. Medium from each bottle was collected, cells were harvested by trypsinization (1 mL 0.25% / 0.02% trypsine/EDTA, Sigma Aldrich, per bottle) and pooled with the medium. Cells were washed twice in phosphate buffered saline (PBS) and pelleted at 1800 rpm for 5 min at room temperature. Pellets were resuspended in fresh media and the number of viable (trypan blue negative), dead (trypan blue positive) and total cells (viable + dead) was counted in five squares at 320× magnification using Neubauer haemocytometer and Leitz-Wetzlar Orthoplan microscope. Cell viability was determined as % of cells that excluded trypan blue stain. The doubling time (td) was calculated according to the following formula:

$$td = \frac{\ln 2}{\mu}$$

$$\mu = (\ln x - \ln x_0)/t$$

where x represents cell number in time t, and x_0 cell number in time t_0.

The same assay was used for determination of viable, dead and total cell number in irradiated samples 24-, 48- and 72 h post-irradiation. The viability index (Vi) of each sample was calculated related to appropriate, unirradiated control, which was used as viability index 1 (100%).

Cell death analysis by flow cytometry. Double staining of cells by Annexin and propidium iodide (PI) enabled estimation of cell viability after irradiation and discrimination between two ways of cell death – apoptosis and necrosis. After trypsinization and centrifugation of cells, approximately 10^5 cells of each sample were mixed with 100 μL of Annexin V-FITC reagent (Travigen Inc., Gaithersburg, MD, USA) containing 5 μg/mL Annexin V-FITC and 5 μg/mL propidium iodide, and incubated at room temperature for 15 minutes in dark and then diluted with 400 μL of binding buffer. Multiparameter measurement of the cell sample in order to detect radiation-induced cell death was performed using a FACS-calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with 488 nm, 15 mW argon-ion laser. Staining of the cells with Annexin V-FITC (Annexin) permitted identification of cells in early apoptosis, while staining of the cells with Annexin and PI permitted quantification of cells in the late apoptosis and necrosis. Data were acquired immediately after staining by analyzing about 20,000 cells/sample. The data were further processed by Becton Dickinson LYSIS II software.

DNA fragmentation assay. This assay was used for confirmation of necrosis, detected by double staining and flow cytometryc analysis. It was performed as previously described with minor modifications (12). Cells were incubated in one volume of digestion buffer (100 mmol/L NaCl, 25 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 8.0, 0.5% SDS and 0.5 mg/mL RNA-se A) for 2 h at 50 °C, followed by the addition of proteinase K (0.6 mg/mL) and digestion was continued overnight. DNA was deproteinised using the phenol/chloroform/isoamil-alcohol reagent, for three times. The aqueous layer was transferred to a new tube and precipitated with one volume of isopropanol and 1/10 volume of ammonium acetate overnight at 4 °C. The DNA pellet was washed three times with ice-cold 95% ethanol and dried at room temperature. The final DNA pellet was resuspended in 20 μL TE buffer (10 mmol/L Tris, pH 7.4, 1 mmol/L EDTA) and the concentration

of DNA was determined spectrophotometrically. Electrophoresis of 4–10 μg of each DNA sample, was carried out for 60 min at 60 V at room temperature on 1% agarose gel containing 1 μg/mL ethidium bromide. Gels were scanned by GelDoc apparatus.

Statistical analysis. For statistical analysis of time- and dose- dependent changes in viability index and flow-cytometric determination, two-way ANOVA was used. If a statistical significance was found, Tukey post-hoc test was used to determine which groups differ from each other. Statistical significance was accepted if $p<0.05$.

Results

Analysis of cell growth and spontaneous cell death in culture

PC-3 cells were plated in 25 cm^2 culture flasks, at the density of 12×10^3 cells/cm^2 (e.g. 3×10^5 cells per bottle), and cell growth, viability and morphology were monitored for 8 consecutive days (*Figure 1*). During the first day post plating the viable cell number increase from 12×10^3 cells/cm^2 to almost 13.5×10^3 cells/cm^2, suggesting that plating efficiency was very high. The log phase of cell growth occurred between 2nd and 6th day post plating. The confluence was reached on the 6th day at the cell density of about 116×10^3 cells/cm^2. Further incubation led to the slightly decrease in cell number. TBE assay showed that approximately 2–8% of all cells were TB positive at all time points, indicating that cell viability was high during the whole experiment. We also calculated PC-3 cell doubling time, which was 32.9 ± 2.8 h under conditions maintained in our laboratory.

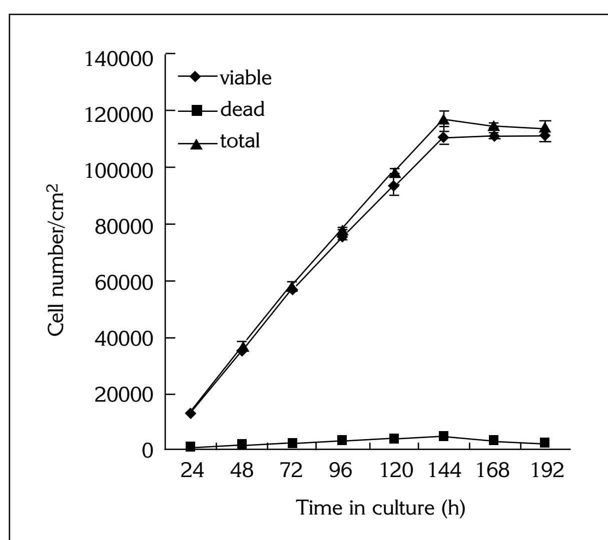

Figure 1. Growth and viability of PC-3 cells in culture determined by TBE assay. Data are the mean SEM from 2 distinct experiments.

Cell growth of irradiated PC-3 human prostate cancer cells

Effects of gamma-ionizing radiation on PC-3 growth were evaluated by TBE assay. The test is convenient for determination of Viability (V) and Viability index (Vi) after radiation treatment. TBE assay was performed 24-, 48- and 72 h after irradiation by 2-, 10- and 20 Gy. The obtained results (*Table I* and *Figure 2*) showed significant decrease in cell number and Viability Index (Vi), comparing with appropriate control, both by dose (F 190.1, $p<0.001$) and time (F 45.2, $p<0.001$) as determined by two-way ANOVA. This effect was most pronounced 72 h after treatment with 20 Gy, when the Vi decreased from 1, established in control, to 0.35. The radiation dose which caused decrease in cell V_i from 1 to 0.5, termed IC_{50} (mitotic cell death dose), was 10.4 ± 0.4 Gy. The dose dependent V_i (e.g. viable cell number) decrease was significant for all experimental points (*Table I*). The statistical differences between groups were analyzed by Tukey post hoc test, comparing irradiated samples with the control from the same time point, and significance was established at $^*p<0.05$. The number of TB positive cells, indicating cytotoxicity (actual cell death), was relatively low (up to 20 %, data not shown), which was in agreement with cytometric quantification of necrotic cells determined after double Annexin V-FITC/PI staining (*Figure 3*).

Table I Number of viable cells in the control and ^{60}Co gamma-irradiated human prostate cancer cells PC-3 measured by TBE assay 24–72 h post-irradiation. Data are the mean ± SEM from 3 distinct experiments. Statistical time- and dose-dependent differences were determined by two way ANOVA and Tukey post hoc test. ** $p<0.01$, *** $p<0.001$.

Dose → Time (h) ↓	control (0 Gy)	2 Gy	10 Gy	10 Gy
24	57.3 ± 1.6	** 43.7 ± 2.8	*** 40.9 ± 4.4	*** 40.9 ± 4.4
48	92.4 ± 4.9	*** 49.9 ± 9.7	*** 46.8 ± 5.9	*** 46.8 ± 5.9
72	84.6 ± 3.9	*** 69.6 ± 1.4	*** 44.7 ± 5.3	*** 44.7 ± 5.3

Radiation-induced cell death analysis by flow cytometry

All samples analyzed by TBE, were also stained by Annexin V-FITC and PI, and analyzed by FACS-Calibur flow cytometer. The results of quantification of cells in different states, such as viable cells, cells in early apoptosis, cells in late apoptosis or necrotic cells, and necrotic cells or cell aggregates, are presented in *Figure 3 A–D*. *Figure 3A* represents a flow cytometry scatter plot and the dots represent cells in different

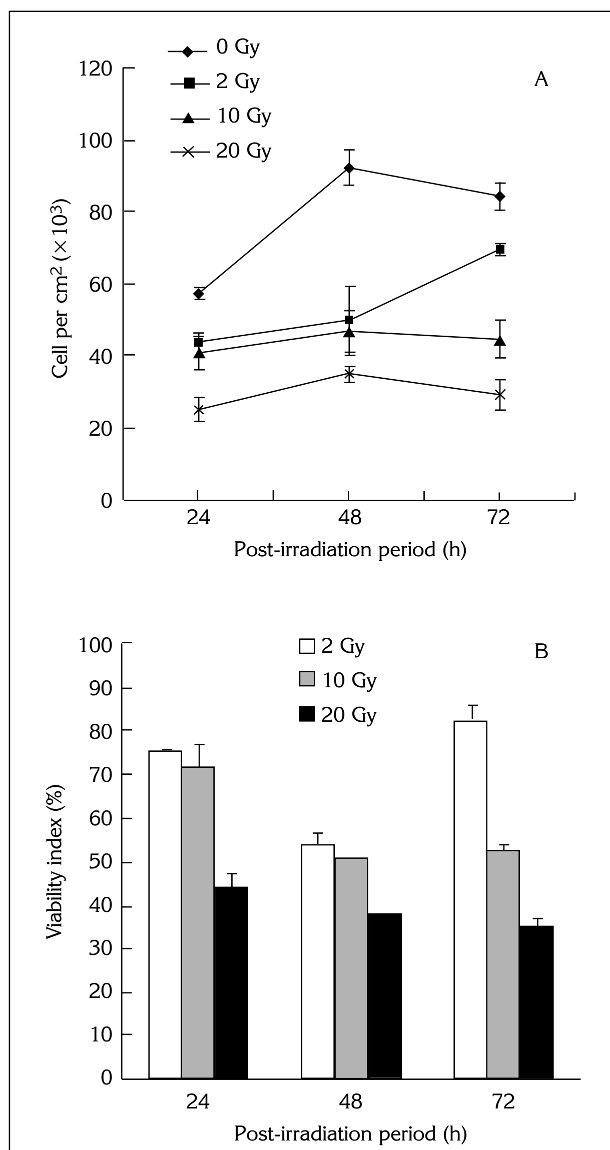

Figure 2. Time course of viable cells number in the control and ^{60}Co gamma-irradiated human prostate cancer cells PC-3 (A) and Viability index calculated according to the related control from the same time point (B) as measured in TBE assay. Error bars represent standard error of the mean (SEM). Data are the mean SEM from 3 distinct experiments.

states, depending on the place in scatter plot. Cells in early apoptosis were Annexin positive, while PI staining indicated late apoptosis or necrosis (*Figure 3A*). As it may be observed from *Figure 3B*, cell viability of the samples decreased with the increase in the radiation dose and with the period of incubation. Radiation-induced decrease of viability showed dose-dependent significance which was judged by two-way ANOVA analysis at $p<0.05$ (F 9.12, $p<0.001$), but not time-dependent significance (F 2.06, $p=0.142$). The Tukey post hoc test, used for comparison of irradiated samples with appropriate control, showed that only radiation dose of 20 Gy induced significant viability decrease, 48 and 72 h post irradiation. The evidence for apoptosis was minimal in either control or irradiated PC-3 cells. With the increase in the radiation dose, the percentage of cells in the state of early apoptosis increased from 0.2% up to 0.8 % (*Figure 3C*), but there were neither time- nor dose-dependent significance (F 1.35, $p=0.33$ and F 1.42, $p=0.33$ respectively).

The radiation dose and time dependent increase in number of PI positive cells in the late apoptosis or necrosis was also observed. The dose dependent increase in necrotic cells was significant (F 7.85, $p<0.001$), but time-dependence was not statistically significant (F 1.9, $p=0.17$). The Tukey post hoc analysis showed significant increase in number of dead cells after irradiation with 20 Gy, 48 and 72 h after treatment (*Figure 3D*).

Electrophoretic analysis of purified genomic DNA from irradiated cells

The genomic DNA from control and irradiated samples, including attached and floating cells, was analyzed on 1% agarose gel containing 1 μg/mL ethidium bromide. As it may be observed in *Figure 4* the initial fragmentation of PC-3 cell DNA to a high molecular size band (>10 Kb) was visible in all samples. In the case of irradiated samples, in addition to the fragmentation of PC-3 cell DNA to a high molecular size band (>10 Kb), smaller fragments (1< Kb) also appeared. The observed smear is most pronounced 72 h post irradiation with doses of 10 and 20 Gy. It correlated well with the highest percent of dead cells obtained by cytometry (*Figure 3D*). DNA ladder was not observed in examined samples, confirming flow cytometric data, that PC-3 do not die by apoptosis after gamma-irradiation.

Discussion

The hormone-independent metastatic prostate cancer is incurable at present. In the lack of efficient chemotherapeutic agents, ionizing radiation therapy still remains as a method of choice for the disease cure. It is known that apoptotic cell death plays an important role in the death of both normal prostate and androgen-dependent malignant prostate tissue following androgen withdrawal. Cancer cell death is leading to a decrease in either glandular or tumor volume, respectively. However, recent data indicate that apoptosis may not be the dominant form of cell death following radio- and chemotherapy in epithelial tissues (7, 13). Disruption of the pathways that lead to apoptosis is one of the major mechanism by which cancer cells become resistant to radiation or chemotherapy (14).

In this paper, we have analyzed ^{60}Co gamma-radiation-induced death of PC-3 human prostate cancer cells. PC-3 cells originates from epithelial cells.

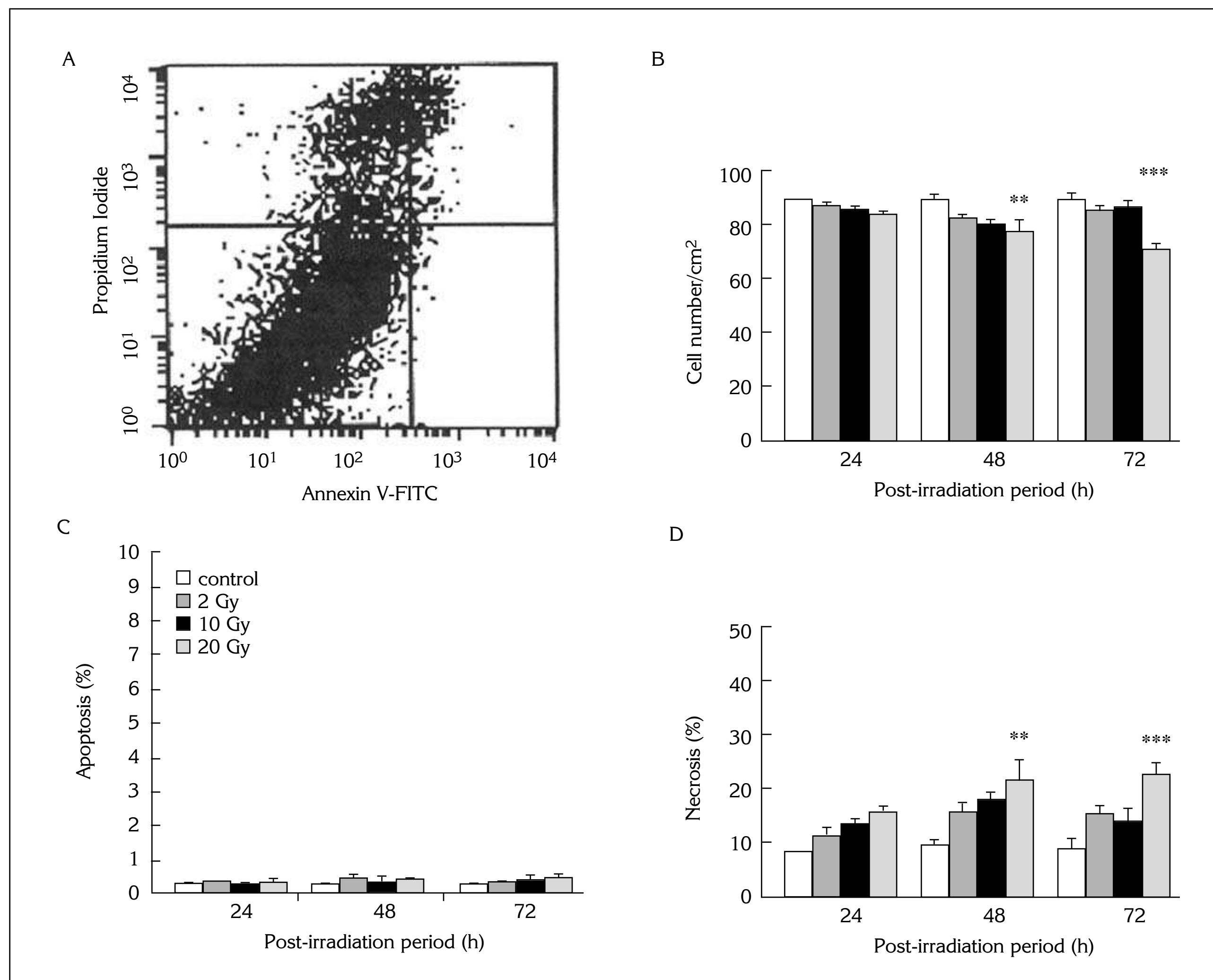

Figure 3. Time course of percent changes in PC-3 cell viability and death after 60Co gamma-irradiation as determined by flow cytometry. Viable cells (A), early apoptosis (B) or late apoptotic/necrotic cells (C). Data are the mean (n=3) and the error bars represent SEM. Statistical differences were determined by two-way ANOVA followed by Tukey post hoc analysis. ** $p<0.01$, *** $p<0.001$.

They are hormone-refractory cells derived from human bone metastasis of prostate adenocarcinoma, representing advanced prostate cancer (15). For these experiments, we chose doses of 2 and 10 Gy, to be representative of the 1.8–2 Gy daily clinical fractions given during curative radiotherapy and the 8–10 Gy single doses given in palliative radiotherapy, as well as the dose of 20 Gy which is in the range of cumulative curative dose for prostate carcinomas (70–80 Gy).

The growth curve of PC-3 cells showed sigmoid-like shape, with the population doubling time about 33 h. The viability of PC-3 cells was 92–98 % throughout the log phase of growth. The culture reach confluence 6th days post plating, at the density of 120×10^3 cells/cm^2, which is considerable less than other epithelial prostate cancer cell line, DU 145 (180×10^3 cells/cm^2), suggesting that PC-3 are bigger than DU 145 (to be published). After day 6th, number of cells slightly decreased, retaining surprisingly high viability (97–98%). Based on data from cell growth curve, all irradiation experiments were performed in the log phase of cell growth. Cells were irradiated with 2–20 Gy from ^{60}Co-source at the dose rate of 20 Gy/h. The irradiated PC-3 cell cultures were followed for three consecutive days *i.e.* through approximately two proliferation cycles. The data obtained by the TBE assay, showed significant decrease in cell Viability index, e.g. in the number of viable cells in irradiated samples relative to the control from the same time point. The process was dependent both on the radiation dose and on the incubation time. On the other hand, the number of TBE positive cells remained relatively low, indicating that radiation caused cell cycle arrest and blocking of cell proliferation, rather than actual cell

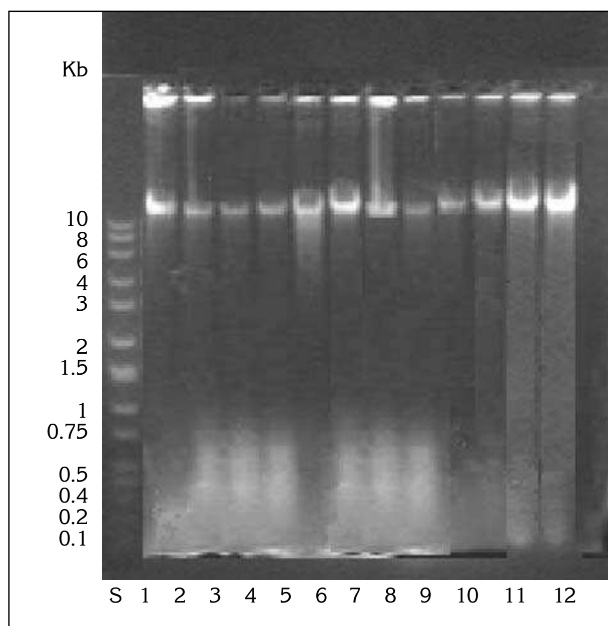

Figure 4. Agarose gel electrophoresis of genomic DNA extracted from ^{60}Co gamma-irradiated PC-3 cells. Lane S: DNA standard molecular size markers; lanes 1–12: genomic DNA pattern of samples irradiated with 0, 2-, 10 and 20 Gy respectively, isolated 24 hours (lines 1–4), 48 h (lines 5–8) or 72 h (lines 9–12) post-irradiation.

death. The obtained data enabled determination of doses which induced 50% of cell growth inhibition (named IC_{50}) and 50% of cytotoxicity, i.e. dose which induced 50% of trypan blue positive cells (EC_{50}). IC_{50} was achieved at the dose of 10.4 Gy, but EC_{50} was higher than applied doses, as 20 Gy induced only 20% of trypan blue positive cells. This indicated that the radiation treatment in the clinically relevant dose interval would predominantly inhibit PC-3 growth rather than induce cell killing. The similar results were obtained for other hormone-refractory epithelial prostate cancer cell line DU 145, derived from brain metastasis (to be published), but in the case of cervix epithelial cancer cells HeLa S3, the same doses of ionizing radiation induced cell death in up to 45% of irradiated cells (16). This comparison suggests that prostate cancer cells are much radioresistant than HeLa S3 cell line.

In order to determine the form of radiation-induced PC-3 cell death two different techniques were used: flow-cytometry analysis of cell morphological features after double staining with propidium iodide and Annexin-V-FITC, and DNA electrophoresis of purified DNA. Simultaneous staining of cells with Annexin V-FITC and propidium iodide enabled distinction of early apoptosis from late apoptosis and/or necrosis. After double staining and flow-cytometryc analysis of control and irradiated samples, the most of dead cells were in late apoptosis or necrosis (Annexin V+, PI+ cells). The early apoptosis (Annexin V+, PI- cells) occurred in insignificant number of cells, less than 1% of total cell number in each sample, and there were no significant changes in percent of apoptotic cells depending on time or dose. On the contrary, the cell necrosis was dose dependent, as determined by two-way ANOVA, and was most pronounced 72 hours post treatment. These results suggest that the prevailing form of ^{60}Co gamma radiation-induced PC-3 cell death was necrosis.

As it was not possible to distinguish the necrosis from the late apoptosis by double staining, it was necessary to perform the gel electrophoresis of purified genomic DNA from PC-3 cells. DNA fragmentation assay confirmed the presence of the necrosis process, showing the absence of DNA ladder characteristic for apoptosis in control (17), as well as in irradiated samples. One explanation for the absence of apoptosis following radiotherapy in PC-3 cell line is that these cells have mutant p53 gene. However, the impairment of other mechanisms necessary for initiation of the apoptotic process is not excluded. Namely, recent investigation showed that gamma-radiation activates acidic sphingomyelinase to produce ceramide, a catabolic product of membrane sphingolipids that is a cell death signal (14, 18, 19). It was suggested that the other epithelial prostate carcinoma LNCaP cells are highly resistant to induction of apoptosis by gamma-radiation due to a defect in ceramide generation (14, 20, 21). Likewise, resistance to apoptosis involves a defect in ceramide generation in the PC-3 prostate cancer cell line (20, 22).

In summary, the obtained results suggests that ^{60}Co gamma-ionizing radiation caused notable human prostate cancer PC-3 cell killing. The irradiated PC-3 cells were mostly dying by necrosis, while apoptotic cell death was negligible. Although within the radiation doses used in this study (2–20 Gy) the EC_{50} *i.e.* 50% of cytotoxicity was not achieved, we found significant cell growth inhibition with IC_{50} of 10.4 Gy. Thus, this *in vitro* study suggests, that the increase in radiation dose may have an important cytostatic effect, rather than eradicating the advanced prostatic carcinoma. It also suggests that, in addition to gamma irradiation, current antitumor strategies should introduce novel cytotoxic adjuvant or radiosensitizers, in order to achieve complete eradication of metastatic human prostate cancer.

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ĆELIJSKA SMRT U OZRAČENIM ĆELIJAMA KANCERA PROSTATE ANALIZIRANA PROTOČNOM CITOMETRIJOM

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Kratak sadržaj: Uprkos značajnom napretku u hemoterapiji kancera, radioterapija ostaje metod izbora u tretmanu metastaziranog kancera prostate. Ovaj rad predstavlja kvantitativnu analizu efekata ^{60}Co gama zračenja na ćelijski rasti i ćelijsku smrt PC-3 ćelijske linije humanog kancera prostate, pri čemu je praćena vremenska (2–72h) i dozna zavisnost (2–20 Gy). Ozračene PC-3 ćelije su uglavnom umirale nekrozom u kasnijem vremenskom intervalu (72h), dok je apoptoza bila zanemarljiva. Vrednost EC_{50} odnosno 50% citotoksičnosti nije dostignuta primenjenim dozama, ali je ustanovljena značajna inhibicija ćelijskog rasta, sa vrednošću IC_{50} od 10.4 Gy. Zaključeno je da povećanje doze može imati značajan citostatički efekat ali da je za kompletno odstranjivanje metastaziranog kancera prostate neophodno uvođenje novih citotoksičnih agenasa ili radiosenzitera kao adjuvanata.

Ključne reči: humani kancer prostate, ćelijska smrt, gama zračenje, protočna citometrija

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