RADIATION INDUCED MULTIDRUG RESISTANCE IN BREAST CANCER - LABORATORY MODEL

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Abstract:Breast cancer relapse is a vast clinical problem. Tumors relapsing after chemotherapy are known to develop multidrug resistance. In this paper the authors investigate the possibility of multidrug resistance cross-induced by radiotherapy. Cytotoxic assay and cell cycle analysis were performed. The results revealed a significant difference in chemosensitivity to doxorubicin between the standard cell line and the radioresistent subline. Differences in cell cycles were also observed.

Keywords: Breast cancer, multidrug resistance, radiation, doxorubicin, toxicity, cell cycle.

1. Introduction

Recovery after initial cancer treatment does not equate with full recovery; fear of tumor relapse looms over both patients and their clinicians.About 30% of all breast cancer patients who are successfully treated at early stages are suffering arelapse accompanied by metastasis [1, 2]. It is down to the fact that tumors usually consist of a mixed population of cells, some cells are therapy-sensitive while others are resistant.Regardless of whether patients underwent chemotherapy or radiotherapy, they might be in need of further treatment of a local relapse.Chemotherapy kills drug-sensitive cells, but leaves behind all of the drug-resistant cells [3]. Cancer cells after treatment with one type of drug can develop resistance towards a wide variety of chemotherapeutics [4] resulting in a multi-drug resistant (MDR) relapse. A major way the cells use to protect themselves against drugs is *via* transport systems or so called “pumps”: ATP-cassette [5] transporter proteins.

A problem that is investigated to a much lesser extent is chemoresistance induced by radiotherapy. It is important from clinical point of view, since some relapse patients are scheduled for a chemotherapy course after radiation treatment to avoid two subsequent radiotherapies [6, 7].Therefore, it is necessary to explore the problem of radio-resistantcells responsiveness to chemotherapy [8].

It was discovered that radiation has a variety of effects oncellular activities, including the cell division cycle, apoptosis, proliferation and senescence [9]. Despite potentially devastating effect, cells can also develop resistance to radiation, may it be intrinsic or induced by radiation. There is more than one mechanism responsible for it, including: high expression levels of anti-oxidant proteins in cancer stem cells [10] and improved DNA repair mechanisms [11]. The possibility remains that some of those traits might render radioresistant cells less sensitive to chemotherapy.

Based on observations drawn from other experiments authors hypothesized that radioresistant MCF7 breast cell subline (later referred to as MCF7/44) is less sensitive to drugs commonly used in cancer therapy (data not shown). The aim of this article is to present preliminary comparative data regarding chemosensitivity for breast cancer cell lines MCF7 and radioresistant subline MCF7/44.

2. Materials and Methods

Cell lines and cell culture

Two breast cancer cell lines used in this study were MCF7 and MCF7/44. MCF7 is a breast cancer cell line, and MCF7/44 is a subline derived from MCF7 (see MCF7/44 construction for details). The cells were grown and routinely maintained in Dulbecco's modified Eagle's medium (DMEM) with supplements (10% fetal bovine serum (FBS) and 1% penicillin-streptomycin) at 37°C supplied in 5% CO_2 in T25 culture flasks placed in a cell incubator, as described by our colleagues [12].

MCF7/44 construction

MCF7 cells were cultured up to 80% confluence. Subsequently, the cells were receiving a daily radiation dose of 2Gy until 44Gy cumulative dose. Before each radiation procedure the cells were washed with PBS and the culture flask was filled with culturing medium to maximum volume. After each procedure the medium was substituted with fresh culturing medium in standard volume for cell culture.

Doxorubicin sensitivity experiment

MCF7 and MCF7/44 cell lines were seeded into two 6-well plates; one plate for each cell line, 200 thousands cells per well. After 24h, doxorubicin (Dox) drug was added to 5 wells on each plate, using different Dox concentrations for each well (1 - 10 μg/ml). One well in each plate was left without Dox addition, as control. Cells with Dox were left for a 24 hour incubating period.
After 24 hours the cells were fixed. Firstly, prepared cells were washed2x with PBS. Afterwards, 1 ml of cold 70% ethanol was added drop by drop onto the cells for 10 minutes, at 4ºC. Subsequently, after discarding the 70% ethanol, 1ml of 96% ethanol was added for further 10 minutes at 4ºC. After the cells were fixed, ethanol was fully removed and crystal violet dye was added. The cells were then incubated with the dye for 15 minutes at room temperature. After the incubation time the crystal violet was removed and the dyed cells were gently washed with PBS. Extraction of the dye was performed with the use of 0.2% Triton X100 solution over the period of 24 hours in 37°C. Absorbance at 630nm of the extracted dye was measured using a spectrometer.

Cell cycle analysis

MCF7 and MCF7/44 cell lines were seeded into two 6-well plates; one plate for each cell line, 200 thousands cells per well. After 24h, doxorubicin (Dox) drug was added to 5 wells on each plate, using different Dox concentrations for each well (1 - 10 μg/ml).One well in each plate was left without Dox addition, as control. Cells with Dox were left for a 24 hour incubating period. Afterwards, the cells were washed in PBS, dissociated from the wells using 0,25% trypsin/EDTA solution. Cells were washed again in PBS. Harvested cells were counted on a Goriaev chamber and stained with propidium iodite (PI) solution according to the cell count (1ml per 1mln cells) for 30 minutes in the dark at room temperature. PI solution composition was as follows: 500ml H_2O; 0,025g PI; 0,01g Ribonuclease A; 0,05g sodium citrate; 0,5ml Triton X-100.
After the staining, cells were visualized on FACS flow cytometer (488nm excitation argon laser, 585/42nm band pass filter).

Statistical analysis

Each experiment was performed at least three times. Results display mean values with standard deviations. P-values were calculated with the use of Student's t-test. Results were considered significant when p-value < 0,05.

3. Results

Cells' sensitivity to doxorubicin

MCF7 and MCF7(44) were compared in the respect of their susceptibility to doxorubicin in Fig. 1.

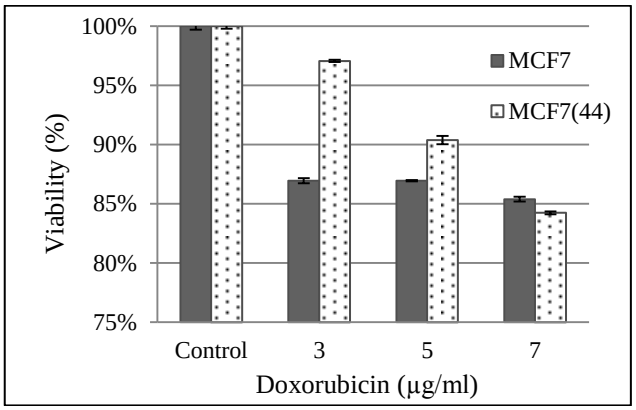

Fig.1.Susceptibility to doxorubicin treatment.MCF7 and MCF7/44 cells were treated with 3, 5 and 7 μg/ml of doxorubicin for 24 hours. Crystal violet assay was carried out to determine cell viability. Results are indicated as percentage of viable cells in reference to control.

Both cell lines react to doxorubicin with a decrease in viability relative to control; (MCF7/44) p-value =0,009 (MCF7/44), p-value =0,012. Differences between the cell lines are of different significance: (3 μg/ml) p-value = 0,008; (5 μg/ml)p-value = 0,786;(7 μg/ml) p-value =0,018.

Cell cycle

Cell cycle analysis was made for MCF7 (Fig. 2) and MCF7/44 (Fig. 3) cells,demonstrating the effect ofdifferent concentrations of doxorubicin on cells'DNA synthesis status.

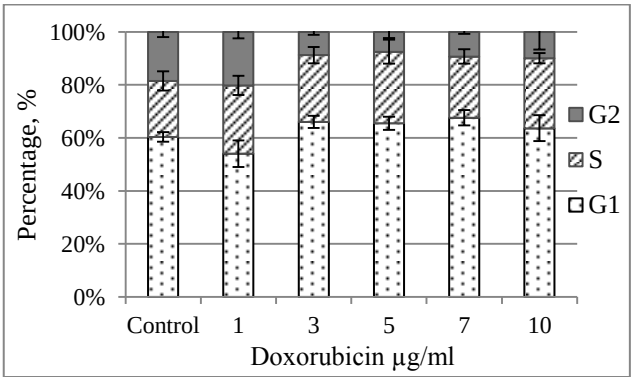

Fig. 2. Flow cytometry analysis of the effect of various doxorubicin concentrations (1, 3, 5, 7, 10 μg/ml) on cell cycle in human breast cancer cell line MCF7.A part of cells was left without adding the drug, as a control.

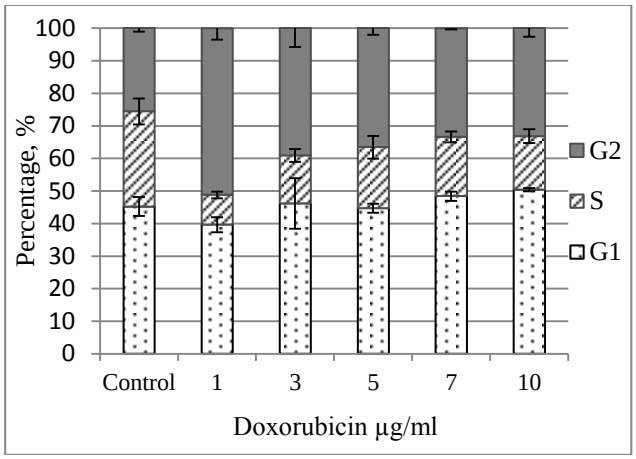

Fig. 3. Flow cytometry analysis of the effect of various doxorubicin concentrations (1, 3, 5, 7, 10 μg/ml) on cell cycle in radioresistantsubline MCF7/44. Control cells did not receive doxorubicin treatment.

Cell cycle analysis showed differences between MCF7 and MCF7/44 cells. The G1/G2 ratios are presented in Table 1 together with corresponding p-values.Cell cycles are detailed in Fig. 4.

Table 1. Cell cycles G1/G2 ratios of MCF7 and MCF7/44 cell linesafter 24h incubation with doxorubicin in various concentrations.

DOX concentrations	Cell line	G1/G2 ratio	p-value
control	MCF7	3,28	0,005
	MCF7/44	1,77	
1μg/ml	MCF7	2,72	0,018
	MCF7/44	0,78	
10 μg/ml	MCF7	4,68	0,075
	MCF7/44	1,53	

4. Discussion

The results of this study demonstrate that both standard MCF7 cells and the radioresistantsubline MCF7/44 are significantly sensitive to doxorubicin (p-value < 0,05) (Fig. 1). The most interesting revelation, however, is that MCF7/44 cells line reacts to lower concentrations of doxorubicin differently than its parent cell line – MCF7. Statisticly significant (p-value < 0,01) weaker sensitivity to 3μg/ml of doxorubicinconfirms the initial hypothesis: radiation therapy can induce chemoresistance in cells with no previous contact with any chemotherapeutics. The difference for 5μg/ml of doxorubicin is statisticly insignificant, but the tendency remains.

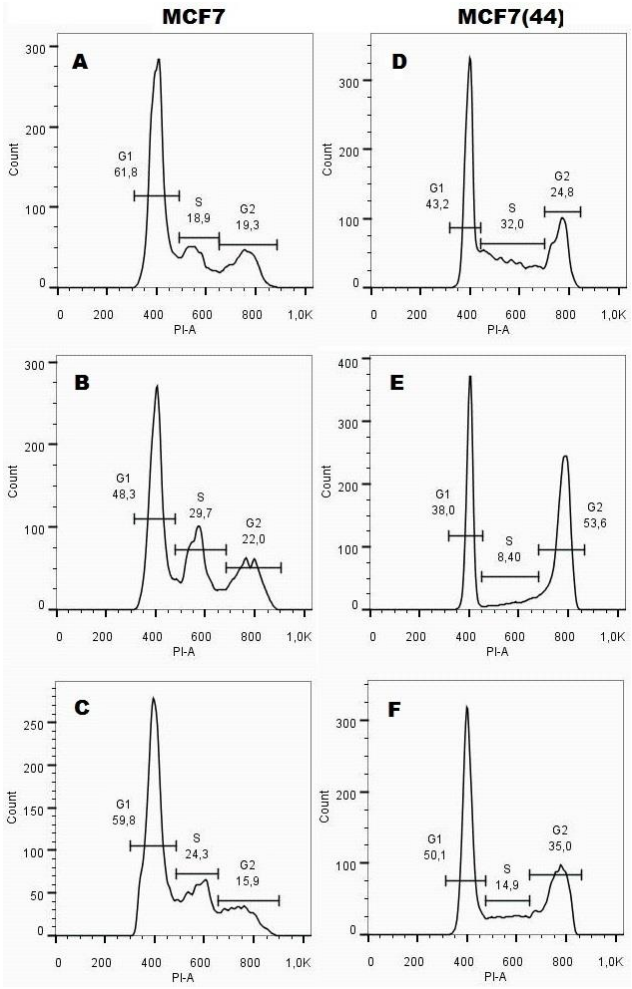

Fig. 4. Comparison of doxorubicins effect on cell cycle of MCF7 (A – control, B – 1μg/ml, C – 10 μg/ml) and MCF7/44 (D – control, E – 1μg/ml, F – 10 μg/ml) cells.

Authors expect that increased number of repetitions will solve the issue of statistical significance. The MCF7/44 cells' chemoresistance seem to diminish with increased drug concentration, when their viability is even lower than that of the parent line – MCF7 (Fig. 1). This suggests that chemoresistance acquired *via* radiation is limited to low drug concentrations only.

This finding is in conflict with previously reported study by Degang Shi *et al*. [6], who found radioresistant lung cancer cells not to differ in their response to doxorubicin when compared to their standard counterparts. This may be due to different cell type or due to random nature of DNA mutations caused by radiation, implying that not every radiotherapy will induce chemoresistance.

If future, more detailed, studies prove these results true, it should be addressed clinicians, since it could possibly directly impact the survival of cancer patients.

To get some understanding of this phenomenon, cell cycle analysis was performed and revealed an increase in G2 phase for MCF7/44 in standard conditions. This explains the more dynamic growth of those cells in cell culture compared to MCF7 cells (data not shown).

Difference between G1/G2 ratios in MCF7 and MCF7/44 cell lines for 10 μg/ml is not statistically significant, borderline significant at best. Authors will address this issue in future experiments with increased number of experiment repetitions.

Low concentrations of doxorubicin induce cell cycle arrest in G2, which is in accordance with the mechanism of action of doxorubicin [13]. MCF7/44 cells shifting to G2 more abruptly than MCF7 cells. This would point to more vulnerability of MCF7/44 cells, however this does not find confirmation in viability data.

Cell cycle for high doxorubicin concentrations is rather confusing, especially when taking into account the lack of statistical significance of this particular result (10 μg/ml). It has been reported, that for high doses of doxorubicin cell cycle arrest in G1 can occur [13], which seems to take place in particular case of MCF7 cells, however the cell cycle of MCF7/44 cell line does not follow this pattern. Instead, the G1/G2 ratioresembles control values, which does not reflect the viability. Authors find those results stimulating and worth of further investigation.

5. Conclusion

Authors found radioresistant MCF7/44 cells to display some level of chemoresistance, contradicting available literature. More studies are needed, if this information is to have an impact on current treatment protocols that involve radiotherapy followed by chemotherapy. Especially, cell cycle analysis is, at its current state, producing more questions than answers, urging for further investigation. Due to the possible implications of confirmed radioinduced chemoresistance phenomenon, further studies are planned.

6. References

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