Ionizing radiation remains an effective tool in cancer therapy. It combines the properties of DNA-damaging agent with high degree of specific spatial action. Nonetheless, considerable differences exist in the outcomes of the radiotherapeutic treatment of tumors of different histological origin [17]. It is known that tumors originating from lymphoid cells (lymphomas, myelomas, leukemias) are generally highly sensitive to radiation effect, while squamous cell carcinomas show intermediate sensitivity, and melanomas and gliomas are the most resistant tumors [8]. Radiation-induced apoptosis was apparent in the radiosensitive (i.e. CH-1 ovarian carcinoma cell line), but not in the radioresistant (i.e. SKOV-3 ovarian carcinoma) cell lines [11]. Better understanding of the molecular mechanisms involved in tumor response to ionizing radiation exposure is important for improving radiotherapy efficiency.

Development of resistance of tumor cells to various anti-cancer drugs is a serious problem appearing during treatment of oncological patients [1, 8]. Different combinations of chemotherapeutic and radiotherapies are usually applied in clinics in order to oppose to that problem. Besides, a cross-resistance of tumor cells to anti-cancer drug(s) and ionizing radiation may also exist. The mechanisms of such cross-resistance are poorly studied, and their understanding can be of an extreme interest when using a combination of chemo- and radiotherapies.

It is known that various stressing agents including ionizing radiation induce different cells to producing bioactive soluble factors, particularly cytokines, both in vitro [18, 28] and in vivo [4, 5, 17]. TGF-β is the most potent inhibitor of proliferation and functioning of normal immune cells and of different cells of the epithelial origin. During early stages of breast cancer development, transformed epithelial cells appear to be sensitive to TGF-β-mediated growth arrest. In contrast, advanced breast cancers were found to be mostly refractory to TGF-β-mediated growth inhibition [26].

Carcinoma cells usually produce large amounts of TGF-β, which may both enhance tumor cell invasion and suppress the immune system cell growth and functioning. That is a consequence of activation of TGF-β latent form produced and deposited into the tumor microenvironment, which supports the clonal expansion of TGF-β-resistant tumor cells [22]. Earlier, we [18] and others [4, 5] found that different cytotoxic agents induced TGF-β production by tumor cells. The level of such production was increased in the anti-cancer drug-resistant cells compared to that in sensitive cells [28]. In addition, cross-resistance of specific tumor cells to growth-inhibiting and apoptosis-inducing actions of the anti-cancer drug cisplatin and of cytokine TGF-β was detected [28]. However, it is poorly known if drug-resistance in tumor cells is accompanied by development of their radio-resistance, and if growth inhibiting cytokine TGF-β can play any important role in that processes.

Here we used human breast carcinoma MCF-7 cell lines differing in their resistance to anti-cancer drug doxorubicin for studying their survival and growth in dependence on the ionizing radiation dose and duration of X-ray in order to check if the cross-resistance to doxorubicin and radiation effects exists. Methods: Determination of cell number and viability using trypan blue (0.1% (w/v)) exclusion method, Western blot analysis of p53 protein expression, biological testing of TGF-β activity, lectinocytocchemical analysis for apoptosis quantitative estimation in unirradiated and irradiated cells of both sublines of MCF-7 cells — sensitive (MCF-7(wt)) and resistant (MCF-7(DOX/R)) to doxorubicin.

Results: It was found that doxorubicin-resistant breast cancer cells were also more refractory to X-radiation-dependent growth inhibition. There were revealed different effects of distinct doses of X-ray on p53 protein expression by cells of both sublines. The level of production of TGF-β was compared in non-irradiated MCF-7 cells and in these cells exposed to X-radiation. It was shown that X-radiation increased TGF-β activity in the conditioned medium of the irradiated cells of both doxorubicin-sensitive and -resistant lines. Conclusions: The results of our study suggest that the biological effects of X-radiation on human breast cancer MCF-7 cells can be at least partly mediated by TGF-β. Taking into account that TGF-β is a potent natural immunosupressor, one may consider that an increased activity of this cytokine can intensify negative effects of X-radiation.

Key Words: MCF-7 human breast carcinoma cells, transforming growth factor β, doxorubicin resistance, X-radiation.
tion of its action. Besides, TGF–β production was measured in both cell lines under the radiation effect in order to check if cross-resistance to doxorubicin and radiation effects may exist.

**MATERIALS AND METHODS**

**Cell lines and culture conditions.** Human breast cancer cell lines differing in their sensitivity to doxorubicin — sensitive (MCF–7 (wt)) and resistant (MCF–7 (DOX/R)) — were originally obtained from cell collection at the Institute of Oncology (Gliwice, Poland). The mink lung epithelial cells of Mv1Lu line were obtained from cell collection at Ludwig Institute for Cancer Research (Uppsala, Sweden). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma Chemical Co., USA) and 50 mg/ml gentamycin (Sigma Chemical Co., USA). The cultured cells were maintained at 37 °C in a humidified (100%) incubator with 5% CO2.

**Irradiation of cells and preparation of conditioned medium.** 4.5 · 10⁵ cells were seeded into 25 cm² tissue culture flasks at least 24 h before the start of the irradiation experiment. 6 h prior to irradiation cells were washed twice with serum-free DMEM, and then placed into 4 ml of serum-free DMEM containing 0.2% bovine serum albumin (BSA, Sigma Chemical Co, USA). Radiation was produced from the X-ray machine “RUM–17” (USSR) at 130 kV, 10 mA, and 18 cm distance from the radiation source to the surface of tissue culture flask, and no filter was used. Irradiation was performed at room temperature at a dose rate of 3 Gy/min. The dose heterogeneity was less than 5%.

For preparation of conditioned medium, the cells were cultured as noted in the experiment protocol and the medium was collected in siliconized tubes, spun at 12 000 g for 10 min to remove cell debris and then flash-frozen at –20 °C.

**Determination of cell number and viability.** The effect of irradiation upon cell growth and viability was studied in 25 cm² culture flasks. At the end of incubation cells floating in the culture supernatant were collected by centrifugation and pooled with adherent cells recovered from the flasks by trypsinization. Total cell number and the proportion of dead cells was determined using trypan blue (0.1% (w/v)) exclusion method and counting cells in the hemocytometer camera under the light microscope.

**Western blot analysis.** MCF–7 cells were lysed in buffer containing 20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 1% Triton X–100, 5 mM EDTA, 1 mM PMSF. After 30 min incubation on wet ice, the cell lysates were cleared up by centrifugation at 12 000 g for 20 min at 4 °C and the supernatants were used as total cellular proteins. Then they were boiled for 5 min with SDS sample buffer (100 mM Tris–HCl, pH 6.8, 0.01% bromphenol blue, 36% glycerol, 4% SDS, 10 mM DTT) and total proteins (30 µg) were subjected to SDS–PAAG electrophoresis. Protein fractions were then electro-phoretically transferred to a nitrocellulose membrane, immunoblotted with polyclonal pan–specific whole-protein anti–p53 antibodies (Sigma Chemical Co., USA) and developed using the enhanced chemiluminescence detection reagents. The same membrane was used for β–actin levels detected by anti–β–actin antibody (Sigma) as an internal loading control.

**Measuring activity of transforming growth factor–β (TGF–β).** For the biological testing of TGF–β activity mink lung epithelial Mv1Lu cells (ATCC CCL–64) were used. The method is based on the ability of TGF–β to inhibit proliferation of these cells. Active form of TGF–β was measured in the conditioned media as previously described by Danielpour D et al [7], with the following modifications: Mv1Lu cells were plated at 96–well tissue culture plates (Sarstedt, Inc., Newton, NC, USA) at 4 x 10⁴ cells/well in 100 µl of DMEM, containing 10% FCS. Before incubation, phase–contrast observations were performed to ensure homogeneous distribution of the isolated cells on plastic surface. 24 h later, the culture medium was removed, and wells were washed twice (2 × 30 min) with serum–free medium. Then, 200 µl of conditioned medium with 5% FCS or 200 µl of DMEM containing 5% FCS and the indicated concentration (0.05–5.0 ng/ml) of TGF–β1 (R&D Systems, Indianapolis, IN, USA) were added to each well for further 72 h. The cells were fixed with trichloroacetic acid (TCA) and stained with 0.4% sulforhodamine B (Sigma Chemical Co., St. Louis, MO, USA) as described by Skehan P et al [25]. The optical density was measured at 492 nm in 96–well microtiter plate reader (Multiskan Plus, Labsystems).

**Lectinocytochemical analysis.** The following plant lectins were used in the experiments: Pisum sativum lectin (PSL), Ricinus communis agglutinin (RCA–120), Tricinium vulgaris agglutinin (wheat germ agglutinin, WGA), Viscum album agglutinin (VAA–1), Galanthus nivalis agglutinin (GNA). Lectins were isolated and purified in our laboratory [16]. Lectinocytochemical analysis was conducted as described [6].

**Statistical analysis.** Experiments were performed in triplicate and repeated 3 times. Significance of difference in a typical experiment was assessed by Student’s t–test. The level of significance was set at 0.05.

**RESULTS AND DISCUSSION**

Two variants of human breast cancer cells — sensitive (MCF–7 (wt)) and resistant (MCF–7 (DOX/R)) to doxorubicin — were subjected to X–irradiation and their growth and survival characteristics were studied. MCF–7 (DOX/R) cells possess acquired resistance to growth–inhibitory and pro–apoptotic effects of the anthracycline antibiotic doxorubicin. Resistance is defined as acquired when tumor cells initially respond to anticancer agents, but become refractory on subsequent treatment cycles. Cytotoxic studies showed that the growth of MCF–7 (DOX/R) cells was not affected by treatment with 4 µg/ml of doxorubicin (concentration used for culturing this type of cells). Various mechanisms have been proposed to explain the development of drug resistance in tumor cells: 1) impaired drug trans-
port inside and from within the cell; 2) increased efficacy of intracellular detoxication mechanisms and DNA repair; 3) increased anti-apoptotic potential of tumor cells. However, none of these mechanisms have been accepted as universal or dominating. It is known from literature that doxorubicin was localised predominantly in the nuclei of MCF-7 (wt), whereas in MCF-7 (DOX/R) cells the drug was barely detectable in perinuclear area. It was found that a single 1.5, 3.0 or 4.5 Gy X-ray dose inhibited growth of both MCF-7 (wt) and MCF-7 (DOX/R) cells tested 48 h after the radiation treatment (Fig. 1, a). In order to determine whether a decrease in cell number was caused by reducing proliferative potential of the cells or by their death, both cell number and proportion of dead cells were counted. As shown in Fig. 1, b, a decrease in cell number after the radiation exposure was caused probably by a reduction in their proliferative capacity and delay in G1 phase of cell cycle rather than by cell death. An arrest in G1 phase is thought to give the cells enough time needed for repairing critical damage before DNA replication occurs, thereby avoiding the propagation of genetic lesions to cellular progeny. The cell cycle can resume once the damage has been repaired, while if the damage is too massive, the cell will undergo apoptosis [21].

The most noticeable differences between MCF-7 (wt) and MCF-7 (DOX/R) cells in number of alive and dead cells were observed at 3.0 Gy dose (see Fig. 1, a). We found that MCF-7 (DOX/R) were more refractory to X-radiation-induced growth inhibition and cytotoxic effect in comparison with MCF-7 (wt) cells. X-radiation effect was shown to be dependent on the duration of cell cultivation after radiation treatment (Fig. 2). It was also detected that MCF-7 (DOX/R) cells grew slower than MCF-7 (wt) cells. Time of cell population doubling was approximately 48 h in MCF-7 (DOX/R) cells in comparison with approximately 24 h in MCF-7 (wt) cells. One of the reason of lower sensitivity of MCF-7 (DOX/R) cells to irradiation may be that slower proliferating cells have slower generation time, so they have more time to repair damages. Usually, more aggressive tumors with higher mitotic rate are character-

![Fig. 1. Effect of X-irradiation on survival (a) and death (b) on MCF-7 cells (48 h after irradiation); *p < 0.05, **p < 0.01 (between non-irradiated and irradiated cells).](image1)

![Fig. 2. Effect of X-irradiation (3 Gy) on survival of MCF-7 cells, sensitive (MCF-7(wt)) and resistant (MCF-7(DOX/R)) to doxorubicin action; *p < 0.05 (between non-irradiated and irradiated cells).](image2)
ized by much more pronounced cell death expression [9]. At the molecular level, the relationship between proliferation and cell death can be explained by participation of the same oncogenes in regulation of both these processes: oncogenes which force cells into cell cycling by omitting cell cycle checkpoints, may also induce apoptosis or "mitotic catastrophe" [20]. But the lower sensitivity of MCF-7 (DOX/R) cells to irradiation can be also explained that the anti-tumor drugs can induce TGF-β, expression which may be accompanied by the development of TGF-β, refractoriness and partial or full specific drug resistance in tumor cells. The latter happens due to mutations in genes coding for TGF-β receptors or in genes for TGF-β signal transduction via Smad proteins.

Taking into account that X-irradiation led to an enhanced TGF-β production by both MCF-7 (wt) and MCF-7 (DOX/R) cell lines, but only MCF-7 (DOX/R) cells were more refractory to TGF-β growth-inhibiting and apoptotic action, we could assume that radiation resistance of MCF-7 (DOX/R) cells was associated with loss of TGF-β signaling. This hypothesis is presently under investigation.

p53 is an anti-oncogene whose protein product stops DNA synthesis in cases when a damaged DNA should be repaired, or switch on the mechanisms of apoptosis when the degree of DNA damage is too massive to be restored [14]. Thus, it was reasonable to study p53 expression in MCF-7 (DOX/R) cells compared to MCF-7 (wt) cells, both in control and after their X-irradiation. To address that question, we used Western blot analysis in which the pan-specific polyclonal anti-p53 antibodies were applied. These antibodies revealed higher level of p53 expression in MCF-7 (DOX/R) cells compared to MCF-7 (wt) cells (Fig. 3, a). It is known that p53 can either mediate or protect cells from apoptosis, depending whether it is wild type or mutant. Mutant p53 proteins are generally more stable than wt-p53 and cells with high p53 protein levels generally express mutated, or at least inactivated forms of p53 [8]. Loss of p53 heterozygosity and a point mutation in the remaining allele of the p53 gene in adriamycin-resistant cells were discovered. This mutation is a splice acceptor site change on the up-stream border of exon 5 and results in p53 protein overexpression [3]. Mutation in p53 plays a role in development of cancer resistance to radiation and chemotherapy [2]. We found that the level of expression of p53 decreased after 1.5 Gy dose of X-ray treatment of both MCF-7 (wt) (p < 0.01) and MCF-7 (DOX/R) (p < 0.001) cells, while after higher radiation doses (3.0 and 4.5 Gy) the increase in p53 expression in MCF-7 (wt) cells and decrease of its expression in MCF-7 (DOX/R) cells was revealed (Fig. 3, b). At the moment, the reasons for such dynamics in p53 expression in the studied cells are not known. It could be speculated that the mechanisms of action of lower radiation dose (1.5 Gy) differ from such mechanisms when cells are subjected to the action of higher radiation doses (3.0 and 4.5 Gy). However, additional studies are needed to get a support for such explanation of the noted differences.

Recently, it was found that cross-resistance between cis-dichlorodiammineplatinum (II) (CDDP; cis-platin) and radiation resistance existed [12]. The murine CDDP-resistant L1210 cell line exhibited cross-resistance to ionizing radiation because of an increased capacity to repair double-strand DNA breaks compared with parental cells.

Our next task was to study the effect of X-irradiation on TGF-β production in both types of MCF-7 cell line. It was found that the production of this cytokine was higher in unirradiated MCF-7 (DOX/R) than that in unirradiated MCF-7 (wt) cells (Fig. 4). Several breast and prostate cancer models in vivo have demonstrated a connection between TGF-β, expression and increased tumorigenicity, increased invasion and drug resistance [28]. It was reported that TGF-β, expression in the irradiated with a dose of 10 Gy right cerebral hemispheres of rats were significantly increased with the lapse of time during 8-week post-radiation period [17].

A single 1.5 Gy, 3.0 Gy or 4.5 Gy X-irradiation dose led to an enhanced TGF-β production by two studied sublines of MCF-7 breast cancer cells. The highest level of activity of TGF-β released into cell culture medium was revealed after 4.5 Gy X-irradiation treatment of MCF-7 (wt) cells (approximately 3-fold higher compared with that in the untreated cells) (see Fig. 4). In the case of MCF-7 (DOX/R) cells, 3.0 Gy X-irradiation dose caused an approximately 2.5-fold elevation in the activity of TGF-β released into cell culture medium (see Fig. 4).

The untreated cells of both sublines of MCF-7 cells continued to proliferate within 96 h, and the activity of TGF-β released into cell culture medium increased proportionally with time of cultivation achieving maximal level at 96 h. In contrast, the X-irradiation-treated cells of both cellular sublines did not change in their number, but attained a comparable level of TGF-β ac-
activity within 24 h and 48 h after radiation treatment (Fig. 5). It can be suggested that tumor cells may gain an ability to activate TGF-β latent form in response to X-radiation exposure, while the untreated tumor cells produce TGF-β latent form but cannot respond to growth inhibiting effect of this cytokine. It was reported that TGF-β immunoreactivity increased in murine mammary gland after whole-body 60Co γ-radiation exposure. The level of active TGF-β, increased significantly within 1 h of irradiation concomitant with a decreased immunoreactivity of latent TGF-β [4, 5]. The ionizing radiation is one of a few exogenous agents known to cause latent TGF-β activation in situ [10].

Fig. 4. TGF-β production (% of control) by MCF-7 cells, sensitive and resistant to doxorubicin action, after X-irradiation; TGF-β activity in unirradiated MCF-7 (wt) cells was 0.328 ± 0.025 ng/ml/450×10⁵ cells/25 cm² flask; *p < 0.05, **p < 0.01, ***p < 0.001 (between intact and irradiated cells), activity within 24 h and 48 h after radiation treatment (Fig. 5). It can be suggested that tumor cells may gain an ability to activate TGF-β latent form in response to X-radiation exposure, while the untreated tumor cells produce TGF-β latent form but cannot respond to growth inhibiting effect of this cytokine. It was reported that TGF-β immunoreactivity increased in murine mammary gland after whole-body 60Co γ-radiation exposure. The level of active TGF-β, increased significantly within 1 h of irradiation concomitant with a decreased immunoreactivity of latent TGF-β [4, 5]. The ionizing radiation is one of a few exogenous agents known to cause latent TGF-β activation in situ [10].

Therefore, the results of our study suggest that the biological effects of X-radiation on human breast cancer MCF-7 cells can be at least partly mediated by TGF-β. Possible sequence of events in the action of X-radiation could be as following: 1) X-radiation, as stressing agent, induces TGF-β production in target tumor cells; 2) TGF-β, causes growth inhibition and/or induces apoptosis in these cells [28]. Taking into account that TGF-β is a potent natural immunosuppressor, one may suggest that an increased activity of this cytokine can intensify negative effects of X-radiation.

Thus, the results of our study suggest that the biological effects of X-radiation on human breast cancer MCF-7 cells can be at least partly mediated by TGF-β. Possible sequence of events in the action of X-radiation could be as following: 1) X-radiation, as stressing agent, induces TGF-β production in target tumor cells; 2) TGF-β, causes growth inhibition and/or induces apoptosis in these cells [28]. Taking into account that TGF-β is a potent natural immunosuppressor, one may suggest that an increased activity of this cytokine can intensify negative effects of X-radiation.

It is known that tumor cells die by apoptosis during radiotherapy or chemotherapy treatment and, thus, monitoring the level of apoptosis may prove usefulness in modulating treatment or in predicting the biological behavior of tumors following treatment [13]. Apoptosis appears to play a pivotal role in control of tumor growth by counterbalancing tumor cell proliferation [8]. Lectins, a group of specific glycoproteins present in plant as well as in animal cells, are used as particular markers at studying cancers and metastatic cell lines [6]. Specific carbohydrate residues of plasma membrane glycoproteins can be detected using lectins due to their binding specificity to carbohydrates. This property of lectins depends on the process of cellular glycosylation [24]. That is why some lectins are widely used in histology and cytology for identification of carbohydrate moieties of membrane components at tumor cell destruction. Recently, it was found that the lectins specific for α-D-mannose (PSL) and β-D-galactose (RCA-120) can distinguish between native and apoptotic murine leukemia L1210 cells and thus, can be used for quantitative estimation of apoptosis in a population of these cells [6].

Lectinocytochemical study of intact and X-irradiated breast cancer MCF-7 cells showed an increased binding of WGA (p < 0.001), PSL (p < 0.01), RCA (p < 0.01), GNA (p < 0.05) lectins with apoptotic MCF-7 (wt) cells compared with intact untreated cells of this subline (Fig. 6; 7, a). Besides, an increased binding of PSL lectin (p < 0.01) with the apoptotic cells of MCF-7 (DOX/R) subline (Fig. 6; 7, b) compared with intact untreated cells of this subline (Fig. 6; 7, a).

Fig. 5. TGF-β production by MCF-7 cells, sensitive (a) and resistant (b) to doxorubicin, after X-irradiation (3 Gy); *p < 0.05, **p < 0.01 (between non-irradiated and irradiated cells).

Fig. 6. Lectinocytochemical analysis of MCF-7 cells: a — Intact MCF-7 (wt) cells stained with WGA lectin; b — X-irradiated MCF-7 (wt) cells stained with WGA lectin; c — Intact MCF-7 (DOX/R) cells stained with PSL lectin; d — X-irradiated MCF-7 (DOX/R) cells stained with PSL lectin.
line was found (see Fig. 6: 7, b). In both cases, apoptosis was induced by 3.0 Gy dose X–radiation treatment. There was no difference between normal and apoptotic MCF–7 (wt) cells with respect to their ability to bind VAA lectin. Thus, X–radiation action is accompanied not only by changes in cellular DNA, but also by changes in expression of the target cell plasma membrane glycoproteins. However, at present it is not clear if these changes in glycoprotein expression are the result of direct X–ray action, or they are a consequence of general apoptotic changes induced by this stressing agent.

In summary, we demonstrated that a single dose of X–radiation caused numerous changes in characteristics of human breast cancer MCF–7 cells. These are: inhibition of cellular growth and increased cell death, induction of p53 protein expression, increased expression of plasma membrane glycoproteins characteristic for the apoptotic cells. Besides, an elevated activity of growth inhibiting cytokine TGF–β was revealed in culture medium conditioned by the X–ray treated MCF–7 cells. Finally, a cross–resistance to anti–cancer drug doxorubicin and X–radiation was observed in the studied human tumor cells. The investigation of the mechanisms which could be responsible for the development of such cross–resistance are in progress.

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REFERENCES


Цель: исследовать влияние одноразового рентгеновского облучения на выживание клеток карциномы молочной железы человека линии MCF-7 с различной устойчивостью к доксорубицину: влияние ионизирующей радиации на апоптоз и продукцию TGF-β.

Методы: число и жизнеспособность клеток определяли с использованием трипанового синего (0.1% (w/v)), а также применяли Вестерн-блот-анализ для выявления экспрессии белка p53, биотестирование активности TGF-β, лектиноцитохимический анализ — для количественной оценки апоптоза в необлученных и облученных клетках линии MCF-7, чувствительных и резистентных к доксорубицину (MCF-7(wt)) и MCF-7(FOX/R) соответственно.

Результаты: установлено, что резистентные к доксорубицину клетки проявляют большую устойчивость к ингибированию роста под влиянием радиационного облучения. Изучен уровень экспрессии p53 в клетках сублинии MCF-7 (DOX/R) по сравнению с таковым в клетках MCF-7(wt) как в контроле, так и при облучении. Уровень продукции трансформирующего фактора роста бета (TGF-β) сравнивали в контрольных и облученных клетках линии MCF-7. Поскольку было установлено увеличение продукции TGF-β как чувствительными, так и резистентными к доксорубицину клетками линии MCF-7, высказано предположение, что данный цитокин по крайней мере частично может выступать в качестве посредника при рост-ингибирующим действием рентгеновского облучения. Выводы: результаты исследования свидетельствуют о том, что биологические эффекты рентгеновского облучения на клетки линии MCF-7 могут быть по крайней мере частично опосредованы TGF-β. Применяя во внимание то, что TGF-β является мощным природным иммунносупрессором, можно предположить, что повышенная активность этого цитокина может усиливать негативные эффекты рентгеновского облучения.

Ключевые слова: клетки карциномы молочной железы человека линии MCF-7, трансформирующий фактор роста β, устойчивость к доксорубицину, рентгеновское облучение.