SKOV-3 and Me45 Cell Response to Cisplatin-Based Chemotherapy: an in Vitro Study

(ovarian cancer / melanoma / cisplatin / heat-shock proteins / glutathione S-transferase π / p53)

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Abstract. We studied malignant melanoma cell line Me45 and human ovarian carcinoma cell line SKOV-3 (resistant to cisplatin, adriamycin and diphtheria toxin), assessing their expression level of p53, HSP70 and glutathione S-transferase GST-π before and after chemotherapy with cisplatin. These proteins may be responsible for the occurrence of chemoresistance in cancer patients. To assess protein expression we used the immunocytochemical Avidin-Biotin-peroxidase Complex (ABC) method. Before application of chemotherapy, proteins p53, HSP70 and GST-π were present in 100 % of the examined melanoma cells. After the treatment, the intensity of the immunocytochemical reaction for p53 increased, whereas the intensity of immunocytochemical staining for HSP70 and GST-π decreased. In SKOV-3 cells, p53 and HSP70 were present in 100 % of the examined cells both prior to chemotherapy and after it. However, the intensity of the immunocytochemical reaction for p53 decreased, while that of HSP70 increased. As regards GST-π, only 5 % of all examined SKOV-3 cells revealed its expression before chemotherapy. Incubation with cisplatin caused an elevation in the number of ovarian cancer cells expressing GST-π up to 50 %. Moreover, the intensity of the immunocytochemical reaction for GST-π significantly increased.

Introduction

Chemotherapy is one of the most common and effective treatments for cancer. The anticancer therapies are mainly based on induction of programmed cell death known as apoptosis – a complex process in which many different pathways are engaged. Cytostatic drugs can influence the expression and activity of apoptosis-related proteins. This may cause induction of apoptosis (which is the main goal of chemotherapy) or its suppression leading to drug resistance.

Ovarian cancer and melanoma are among the most dangerous tumour types of and difficult to early diagnose and treat. The recurrences occur in most ovarian cancer patients (55–75 %) within two years. In addition, many of these patients die because of a low efficiency of second- and third-line chemotherapy (Miedzińska-Maciejewska and Bodnar, 2004). Malignant melanoma, the most common cancer of the skin, is characterized by rapid formation of metastases (Gurtowska and Kloskowski, 2009). Among metastatic melanoma patients the overall 5-year survival is less than 10 % (Li et al., 2000) and the median survival of these patients amounts to only 6–10 months (Mackiewicz and Kwinta, 2010). These two types of cancer are mainly treated with platinum-based chemotherapy (Czernek et al., 2009).

The effectiveness of the cytostatic drugs may be affected by many proteins including p53, GST-π and HSP70, engaged in the cellular defence mechanisms. Features of these proteins such as their expression level, conformation and changes caused by mutations in genes coding for them influence the cell response to medical treatment and the way in which the cells die: apoptosis, necrosis or autophagy.

p53 is a transcription factor and functions as a tumour suppressor protein by regulating the cell cycle. This pro-
tein is activated in response to stress conditions such as hypoxia or DNA damage. Its activation leads to inhibition of the cell cycle progression, which allows repair of cellular injuries or, when the repair is not possible, directs the cell into the apoptotic pathway (Sznarkowska et al., 2010). The functioning of p53 is associated with the activation of transcription of pro-apoptotic genes (including puma, bax, apafl, noxa) and its translocation to the mitochondria (Stepeń et al., 2007). The translocation increases the mitochondrial membrane permeability and causes cytochrome c release, p53 also acts as an inhibitor of anti-apoptotic genes, among which bcl2, bclX, CCNB1, MAP4 and BIRC5 should be mentioned (Stepeń et al., 2007; Sznarkowska et al., 2010).

Mutations of TP53, gene coding for p53, are among the most frequent causes of human cancer (Bai and Zhu, 2004). Detailed analysis of p53 mutations can be used to diagnose, measure or predict progression of the disease (Hollstein et al., 1994).

GST-π (EC 2.5.1.18) is widely distributed in human organs: lung, bladder, colon, prostate, testicles, erythrocytes and lymphocytes. In pathological states such as carcinogenesis, the expression level of GST-π fluctuates in the affected tissues. Changes of its synthesis level to the mitochondria (Stępień et al., 2007). The translocation of GST-π out in different ways, inter alia: genome protection, in the mitochondria (Saenz-Santamaria et al., 1995; Iersel, 1996).

The main purpose of our work was to evaluate the expression levels of three cancer-implicated proteins, p53, GST-π and HSP70, in human malignant melanoma (Me45) and cisplatin-resistant human ovarian carcinoma (SKOV-3) cell lines under normal conditions and after cisplatin treatment. We also focused on determination of the involvement of these proteins in the drug resistance phenomenon.

### Material and Methods

#### Cell culture

The studies were performed using the SKOV-3 line – human ovarian carcinoma cells resistant to diphtheria toxin, cisplatin and Adriamycin (ATCC, Lomianki, Poland) and Me45 line – human malignant melanoma cells derived from the metastatic lymph node (kindly supplied by Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology Gliwice Branch).

Cells were grown in polystyrene flasks with 25 cm² cell culture surface (Falcon, Becton Dickinson and Co., Franklin Lakes, NJ) as a monolayer in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, St. Louis, MO) containing 2 mM L-glutamine, 10 % foetal bovine serum (FBS, Sigma-Aldrich) and 50 μg/ml streptomycin (Sigma-Aldrich) at 37 °C in 5% CO₂. For the experiments the cells were removed by trypsinization (trypsin 0.25% and EDTA 0.02%; Sigma-Aldrich) and washed with PBS.

#### Immunocytochemistry

Cells were grown in 8-well plates (Diagnostic Microscope Slides, Erie, Thermo Scientific’s ColorFrost™, Portsmouth, NH). For evaluation of protein expression (GST-π, HSP70 and p53), the Avidin-Biotin-peroxidase Complex (ABC) technique was used according to the enclosed kit protocol (Universal LSAB™ Kit/HRP, Rabbit/Mouse/Goat, Dako North America Inc., Carpinteria, CA). The following primary antibodies were used: monoclonal anti-p53 antibody (1:100, p53(DO-1)sc-126, Santa Cruz Biotechnology, Dallas, TX), monoclonal anti-HSP70 antibody (1:100, HSP70(3A3)sc-32239, Santa Cruz Biotechnology) and polyclonal anti-GST-π antibody (1:100, GST(Z-S)sc-459, Santa Cruz Biotechnology). For imaging, an Olympus BX51 light microscope was used (Olympus, Tokyo, Japan). Quantification of the p53-, GST-π- or HSP70-positive cells was performed by taking digital overview images of three ran-
domly selected areas at a low power magnification (10×). In each of these images cells were manually calculated (100 cells per area). The p53-, GST-π- or HSP70-positive cells were expressed as percentage of all counted cells.

**Results**

**p53 expression level**

The expression of p53 was observed in 82 % of examined Me45 cells. The cisplatin treatment induced p53 expression in all cells (Fig. 1, Table 1). The intensity of immunocytochemical reaction before cisplatin therapy was determined as low and medium, whereas after addition of cisplatin it shifted toward high and was accompanied by changes in the cell morphology.

The presence of p53 was confirmed in all examined SKOV-3 cell before cisplatin treatment (Fig. 1, Table 1). The intensity of immunocytochemical reaction was mainly high or, in some cells, medium. The cisplatin application caused reduction in the intensity of immunocytochemical reaction and did not lead to any morphological abnormalities in these cells.

**GST-π expression level**

The Me45 cells are characterized by high expression of GST-π. This was reflected in our experiment in which the intensity of immunocytochemical reaction was high in all examined Me45 cells. Cisplatin treatment of these cells caused reduction in the intensity of immunocytochemical reaction and was determined as medium (Fig. 2, Table 2). Chemotherapy of Me45 cells did not cause any morphological changes.

![Fig. 1. Immunocytochemical evaluation of p53. The protein was detected using the immunocytochemical ABC method. A. Me45 cells before cisplatin treatment, B. SKOV-3 cells before cisplatin treatment, AI. cisplatin-treated Me45 cells, BI. cisplatin-treated SKOV-3 cells.](image1)

![Fig. 2. Immunocytochemical evaluation of GST-π. The protein was detected using the immunocytochemical ABC method. A. Me45 cells before cisplatin treatment, B. SKOV-3 cells before cisplatin treatment, AI. cisplatin-treated Me45 cells, BI. cisplatin-treated SKOV-3 cells.](image2)

**Table 1. The level of p53 in cell lines SKOV-3 and Me45 before and after chemotherapy with cisplatin. The intensity of the immunocytochemical reactions: – no staining, + low, ++ medium, +++ high.**

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<th>Me45</th>
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<td>10 µM cisplatin</td>
<td>+/−</td>
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<td>Control cells</td>
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<td>% of positively stained cells</td>
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**Table 2. The level of GST-π in cell lines SKOV-3 and Me45 before and after chemotherapy with cisplatin. The intensity of the immunocytochemical reactions: – no staining, + low, ++ medium, +++ high.**

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<td>Control cells</td>
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<td>Localization of immunocytochemical reaction</td>
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Before addition of cisplatin, GST-π expression was observed in less than 5% of the examined SKOV-3 cells. The intensity of the reaction was low (Fig. 2, Table 2). Cisplatin treatment caused an increase in GST-π expression, which was detected in 50% of the examined SKOV-3 cells. The intensity of immunocytochemical reaction was determined as low or medium. After chemotherapy, SKOV-3 cells did not show any morphological abnormalities.

HSP70 expression level

The expression of HSP70 was confirmed in all examined Me45 cells. The intensity of immunocytochemical reaction was high. Cisplatin treatment did not abolish HSP70 expression in the tested cells; however, the intensity of immunocytochemical reaction decreased and was determined as low or medium (Fig. 3, Table 3). Chemotherapy did not lead to morphological changes in Me45 cells.

In untreated SKOV-3 cells, expression of HSP70 occurred in less than 5% of all examined cells. The addition of cisplatin resulted in an increase of HSP70 expression, which was present in all examined SKOV-3 cells. The intensity of immunocytochemical reaction was determined as medium or high (Fig. 3, Table 3). No morphological abnormalities were detected in these cells as well.

Table 3. The level of HSP70 in cell lines SKOV-3 and Me45 before and after chemotherapy with cisplatin. The intensity of the immunocytochemical reactions: – no staining, + low, ++ medium, +++ high.

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Discussion

In our study we observed changes in the expression level and distribution of p53 accompanied by abnormalities in cell morphology after cisplatin-based chemotherapy of Me45 cells. In comparison to the initial p53 level (which we determined as medium), the treatment caused an increase of its synthesis (in 82% of examined cells). The distribution of p53 shifted from close to the nucleus to cytoplasmic (Fig. 1, Table 1). The observed changes in the expression level of p53 in Me45 cells from medium to high suggest that there is no p53 overexpression. Moreover, cell disruption after cisplatin treatment may indicate proper activation of p53-dependent apoptosis.

Malignant melanoma belongs to chemotherapy-resistant cancer in which apoptosis is induced with difficulties (Pawlicki and Ziobro, 2001; Gurtowska and Kloskowski, 2009; Mackiewicz and Kwinta, 2010; Skórzewska et al., 2011). In the therapy of malignant melanoma, dacarbazine is the most frequently used drug with the response rate about 20% (Skórzewska et al., 2011). The platinum-based therapy is mostly used as additional treatment or as second-line chemotherapy. Unfortunately, the effectiveness of these drugs is still unsatisfactory (Gurtowska and Kloskowski, 2009).

Degradation of cells that was observed in our experiments may suggest that cisplatin is an effective drug in melanoma therapy. Noteworthy, overexpression of p53 resulting from mutation of TP53 occurs relatively rarely in melanoma patients (only 0–30%) (Pawlicki and Ziobro, 2001; Faran et al., 2004). Moreover, excessively accumulated p53 is a wild form with disrupted function (Kichina et al., 2003). The accumulation of p53 in melanoma cells derives, inter alia, from overexpression of other genes such as CDNK2 coding for a protein that inhibits MDM2-dependent p53 degradation. Functional abnormalities of wild-type p53 are probably a consequence of its excessive phosphorylation, overexpression of proteins suppressing transcription of genes associated with p53 activity, mutation of p53 target genes, or loss of adapter proteins (Avery-Kiejda et al., 2011). This may lead to abnormal cell proliferation and an improper response to apoptosis-inducing treatment (Avery-Kiejda et al., 2011).

We also confirmed overexpression of p35 in the cisplatin-resistant SKOV-3 ovarian cancer cell line (Fig. 1, Table 1). The accumulation of p53 before cisplatin treat-
ment may suggest the presence of mutation in the TP53 gene. This is very plausible since mutations in the gene encoding p53 are common in ovarian cancers (Milner et al., 1993). Using the single-strand conformation polymorphism (SSCP) technique, Milner et al. (1993) confirmed TP53 mutation in 44% of ovarian cancer patients examined. Mutation in TP53 may be a primary phenomenon associated with ovarian tumorigenesis, or a secondary event occurring during chemotherapy (Miedzińska-Maciejewska and Bodnar, 2004). This may be the cause of the drug resistance development. However, according to Siddik (2003), the resistance to cisplatin occurs regardless of the TP53 status. Siddik (2003) indicated many examples of cells with the mutant forms of p53 that were more sensitive to cisplatin than those with the wild-type form of p53. This may also be connected with the functioning of p53 partner proteins and the presence of other p53-independent pathways of apoptosis induction. It is worthy of note that p35 overexpression usually indicates a reduced survival, but it is not always directly related to the chemotherapy response (Shiga et al., 1999).

In the case of malignant melanoma (Me45 line), in our study we confirmed a high level of GST-π expression (Fig. 2, Table 2). This is in perfect agreement with the results obtained by other researchers who also found a high level of the GST-π isoform (compared to the μ isoform and sporadically occurring α isoform) (Schadendorf et al., 1995; Moral et al., 1997). It is worthy of note, however, that in comparison to other carcinoma cell lines, such as H358 (human bronchoalveolar carcinoma cells), A459 (lung cancer cells), Hec116 (colon cancer cells), FN-H296 (adrenal carcinoma cells) and MCF10A (mammary carcinoma cells), the level of GST-π expression in the Me45 line is low and compared to GST-π expression in mammary carcinoma cells it amounts to only 0.299 (Slonchak et al., 2011). Moreover, Schadendorf et al. (1995) observed that the increase in the GST-π expression level was accompanied by a broad spectrum of changes from mild skin lesion to metastatic malignant melanoma.

In a study of three melanoma cell lines (GLL19, Me8, JUNO), Benathan et al. (1992) demonstrated that well-differentiated cancer cells (in comparison to poorly differentiated melanoma) are characterized by higher levels of GST-π activity and DOPA oxidase, and a higher content of GSH. The lowest efficacy of chemotherapy was observed in the poorly differentiated Me8 cell line. This may be caused by the competition for GSH between the chemotherapeutic agent (melphalan) and melanin synthesis intermediates – the lower the production of melanin, the greater the amount of GSH that can conjugate to cytostatic drugs. Moreover, although Schadendorf et al. (1995) indicated that metabolic alterations in the GSH level do not play a significant role in melanoma chemoresistance, they cannot be excluded. Generally, a high level of GST-π is considered as an indicator of cytostatic drug resistance in cancers treated with cisplatin, doxorubicin and alkylating compounds (Kulbacka et al., 2008).

Our results indicated cisplatin resistance in Me45 cells in which the GST-π level after therapy was not reduced and no morphological abnormalities of cells were observed (Fig. 2, Table 2). We assume that in this case the occurrence of the drug resistance may be associated with the MRP protein. The MRP protein acts as a pump in the extracellular transport of drugs and reduces their content in the cell. A similar function is performed by P-glycoprotein, which however was not demonstrated to participate in the occurrence of drug resistance in melanoma. Chemoresistance may also be connected with qualitative and quantitative changes of topoisomerase II, protein that is involved in mitosis. It has been shown that the decrease of MMR, involved in the repair of mismatch damage, is also responsible for the ineffectiveness of chemotherapy (Helmbach et al., 2001).

In contrast to other reports, in our study ovarian cancer cells (SKOV-3) did not show overexpressed GST-π (Fig. 2, Table 2) (Murphy et al., 1992; van der Zee et al., 1992; Green et al., 1993; van der Zee et al., 1995; Wrigley et al., 1996; Lu et al., 2011). Silvestrini et al. (1998) observed a small proportion of GST-π-positive patients at the level of 25%, of whom only 20% revealed the presence of the enzyme in their cells. Moreover, Kolwijk et al. (2009) observed GST-π overexpression in the fluid from ovarian cysts (oCF). The detected GST-π isoform was predominantly cytoplasmic and the presence of the enzyme in the nucleus was believed to be a result of its diffusion (van der Zee et al., 1995; Wrigley et al., 1996).

Our findings suggest the occurrence of the drug resistance in ovarian cancer cells because of GST-π overexpression after the therapy (Fig. 2, Table 2). van der Zee et al. (1992, 1995) noticed that the GST-π level did not affect the efficacy of platinum-based treatment and chemoresistance. Also, Murphy et al. (1992) did not support the hypothesis in which the reduction of the GST-π level or activity would help increase the chemotherapy effectiveness. In spite of these reports, the involvement of a high level of GST-π in the development of chemoresistance has been postulated (Green et al., 1993; Lu et al., 2011).

Some researchers observed a correlation between the high level of GST-π and poor prognosis in ovarian cancer (Green et al., 1993; Kolwijk et al., 2009; Lu et al., 2011) as well as in other cancers treated with cisplatin-containing cytostatic drugs (CCDP/5-FU, CCDP/paclitaxel) (Shiga et al., 1999). The increase of cisplatin resistance in ovarian cancer is associated with the intracellular GSH level, which is engaged in the DNA repair mechanism, protein protection, but also participates in the drug metabolism (including cytostatics) (Godwin et al., 1992). In spite of the diverse GST-π expression level and activity, the metabolism of GSH associated with the activity of the examined protein should be taken into consideration in cancer therapy.
In our study we observed accumulation of HSP70 in the Me45 cell line (Fig. 3, Table 3). HSP70 plays an anti-apoptotic and cell-protecting role also toward cancer cells, which due to excessive proliferation and abrupt differentiation are prone to metastasis (mainly in the lymph nodes). We observed that the application of cisplatin led to a significant decrease of immunocytochemical reaction of HSP70 in comparison to its initial level (Fig. 3, Table 3). This suggests activation of cancer suppression factors (p53, Apaf1) and directing of cells toward apoptosis (mitochondrial/lysosomal), and thereby implies effectiveness of the applied treatment (Każmierczuk and Kiliańska, 2010).

In our study cisplatin treatment did not reduce the high level of HSP70 expression in SKOV-3 cells (Fig. 3, Table 3). Similar observations were made by other researchers (Gong et al., 2010; Sirotkin and Bauer, 2011), who suggested application of this protein in a novel anticancer approach. This strategy assumes use of specific anti-HSP70 vaccines directed against the plasma membrane fraction of HSP70. The vaccines should induce an intensive immune response against transformed cells overexpressing HSP70 to eradicate cancer (Gong et al., 2010). Gong et al. (2010) investigated HSP-based vaccines directed against the plasma membrane fraction of HSP70. The vaccines should induce an intensive immune response against transformed cells overexpressing HSP70 to eradicate cancer (Gong et al., 2010).

Another alternative treatment of cisplatin-resistant tumours encompasses the use of farnesyl transferase inhibitors (FTIs) and manumycin, which inter alia block one of the crucial stages of p21/ras oncogenic activation. Hu et al. (2002) successfully applied these inhibitors in his study of HSP70-overexpressing ovarian cancer cell line (OVCAR3). FTIs are used in the treatment of human cancer with RAS mutation.

Hendrickson et al. (2012) indicated that the resistance of cancer cells (epithelial ovarian cancer cells and primary peritoneal cancer cells) to platinum derivatives is associated with factors such as Her2, Akt, IGF, InsR, c-Raf and Chk1, which are client proteins for both HSP70 and HSP90. An excessive amount of HSPs in cancer cells indicates resistance to cisplatin in this type of tumours, which is in perfect agreement with the results of our research and shows the need to seek alternative methods of treatment. This again confirms that the excessive amount of HSPs in tumour cells may be a useful marker.

Conclusions

Cancer cells are characterized by excessive proliferation that is not accompanied by differentiation. Their abnormal metabolism may be a consequence of the loss of cell cycle control associated with mutation in genes coding for control proteins and/or inability to modulate cell death processes such as apoptosis, necrosis and autophagy. Three proteins examined in our study, p53, GST-π and HSP70, belong to factors controlling the mentioned processes. The impact of cytostatic drugs on the processes responsible for malfunctioning of control proteins should be further studied. Broad knowledge about the cytostatic drug properties and action will allow us to elaborate effective and safe cancer therapies, as well as modulate metastasis and multidrug resistance.

References


